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(54) Title: METHODS FOR INDUCING IMMUNE RESPONSIVENESS IN A SUBJECT

(57) Abstract

The present invention provides improved methods for extracorporeal blood treatment, such as photochemotherapy, and related compositions are provided. The improved method involves introducing dendritic cells into the extracorporeal blood stream during agent treatment to further enhance the subject's immune system response to antigens present in the peripheral blood. The present invention further provides methods of identifying agents for use in the extracorporeal treatment of blood based on the ability of the agent to increase MHC expression.

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METHODS FOR INDUCING IMMUNE RESPONSIVENESS IN A SUBJECT

Technical Field

The present invention relates to improved methods for inducing an immune response in a subject to cells or tissues, particularly tumor cells and/or T or B-cells that cause an autoimmune disorder. The present invention specifically provides methods and compositions for the extracorporeal treatment of blood and for administration of an extracorporeally treated blood mixture to a subject, to induce an immune response to cells or tissues that express a target antigen. In particular, the methods include using extracorporeal treatment agents, such as photochemotherapeutic agents, that increase MHC Class I peptide expression, and using/adding dendritic cells and/or other antigen presenting cells to the extracorporeal blood stream during treatment with such agents.

Background Art

Immune system responses may be classified as humoral or cell-mediated. A humoral response is mediated by B lymphocytes in the form of freely diffusible antibody molecules. A cell-mediated response is mediated by specifically reactive lymphocytes, such as T lymphocytes ("T cells"). T cells react with foreign antigens via surface receptors that are distinctive for each T cell clone. The T cell surface receptors generally are composed of two disulfide-linked protein chains having unique amino acid sequences (Edelson, R., *Annals of N.Y. Acad. of Sciences* 636:154-164 (1991)). The physical properties of these receptors confer specific binding capabilities and permit each of the several million clones of T cells in an individual to operate independently.

T cells function in the regulation of an immune response via recognition by the immune system of the T cell surface receptor. In the initiation of an immune response, the T cell receptor is capable of recognizing a particular antigen only when it is associated with a surface marker on an antigen presenting cell, such as a dendritic cell. These surface markers most commonly belong to a group of molecules known as the major histocompatibility complex (MHC). Binding of the T cell receptor to the

antigen on the antigen presenting cell induces changes in the T cell, which changes collectively initiate a cascade of events leading to the cell-mediated immunologic response.

Cutaneous T cell lymphoma (CTCL) is an immune system disease that is caused by a massive expansion of a single clone of aberrant T cells. Extracorporeal photochemotherapy "photopheresis" for the treatment of cutaneous T cell lymphoma has been described (Edelson, R., *Scientific American* 256(8):68-75 (1988); Edelson, R., *supra*. (1991)). Photopheresis treatment involves isolating the subject's white blood cells (including the T cells), irradiating the cells in the presence of a photoactivatable agent (8-methoxypsoralen, "8-MOP") and reinfusing the damaged cells. The 8-MOP is activated by the ultraviolet light to form a transiently energized molecule capable of photomodifying cellular DNA. This therapy reportedly results in selective destruction of the malignant T cell clone. It is believed that exposure of as little as five percent of the members of the malignant T cell clone to the 8-MOP/irradiation treatment, followed by return of the irradiated, damaged cells to the subject, elicits a specific response to the aberrant T cells that is mediated by the T cell surface receptors, i.e., the damaged cells of the malignant clone in effect prime the immune system to specifically destroy the untreated remainder of the aberrant clone. Photopheresis also has been used for the treatment of several autoimmune disorders, including pemphigus vulgaris, systemic sclerosis, rheumatoid arthritis, HIV infection and rejection of transplanted organs.

U.S. Patent Nos. 4,321,919; 4,398,906; 4,428,744; and 4,464,166, all issued to Edelson, describe photopheresis methods for treating the blood of a diseased subject where the disease-producing blood cells have been naturally stimulated as a consequence of the disease state, either as an immunologically reactive state or a malignancy. Specifically, the methods involve treating such naturally stimulated specific human blood cells with a dissolved photoactivatable drug, such as a psoralen which is capable of forming photoadducts with the DNA of the diseased cells, in the presence of ultraviolet or visible light irradiation. Following extracorporeal irradiation, the damaged lymphocytes are returned to the subject. The damaged lymphocytes are

cleared from the subject's system by natural processes, but at an accelerated pace, presumably because of disruption of cell membrane integrity, alteration of the DNA within the cells, or related modifications.

More recently, methods and pharmaceutical compositions for specifically modifying an immune response to a specific antigen have been reported. These methods include treating an antigen-presenting cell to enhance expression by the cell of empty major histocompatibility complex molecules, followed by reacting the treated antigen presenting cell with an antigen extracorporeally in the presence of a photoactivatable agent and irradiation to form an antigen-associated antigen presenting cell. (See, e.g., PCT application number US93/11220, publication number WO 94/11016). None of the references and/or patents disclosed herein describes extracorporeal blood treatment methods that increase immune reactivity to specific target antigens and methods that can be used to augment existing photopheresis methods. Accordingly, there is still a need for improved methods and pharmaceutical compositions for inducing an immune response to a target antigen, for methods of preparing disease-associated antigen preparations which are specific for a subject and methods for augmenting existing photopheretic methods.

Summary of the Invention

The methods and compositions of the present invention are based on the identification that: 1) current agents used in photopheretic methods to induce an immune response to one or more target antigens are effective because they increase the level of MHC expression in the treated cell, 2) blood, containing disease effector cells, that is extracorporeally treated in known photopheretic methods results in the transport of disease associated antigens to the surface of the treated cells as weakly bound antigens to MHC molecules, 3) known photopheretic methods can be substantially improved by adding dendritic cells, or other antigen presenting cells, to the treated blood prior to re-infusion and 4) isolated and cultured dendritic cells can be used to boost a subject's immune system response in most context where an immune system response in desired.

Based on these observations, the present invention provides: 1) methods and pharmaceutical compositions for preparing a disease-associated antigen preparation; 2) methods for inducing an immune response in a subject using extracorporeal treatment of blood; 3) methods for augmenting existing extracorporeal blood treatment methods, such as photochemical therapy; 4) methods for identifying agents that can be used in the extracorporeal treatment of blood; 5) methods to optimize extracorporeal blood treatment methods; and 6) methods for augmenting existing immunotherapeutic methods.

One embodiment of the present invention is based, at least in part, on the discovery that disease-associated antigens contained in unfractionated blood that has been subjected to treatment with an agent that increases MHC Class I expression (such as in photopheresis using a photoactivated chemical), can be presented by exogenously added dendritic cells *in vivo* to elicit an antigen-specific immune response. More particularly, one embodiment of the invention provides methods and compositions for an improved method for extracorporeal blood treatment in which dendritic cells are introduced into the treated blood during the extracorporeal treatment, such as during photopheresis. Also provided are methods for inducing immunologic tolerance to autologous or exogenous antigens and compositions useful in suppressing clinically undesirable immunologic reactions.

In general, these methods rely on the use of dendritic cells. Dendritic cells, preferably peripheral blood dendritic cells, or other antigen presenting cells, are first removed from a subject and cultured *in vitro*. The cultured dendritic cells can be added to an extracorporeal treated blood sample, such as that described above, to increase the degree of immune response obtained. In addition, cultured dendritic cells can be added in combination with a subunit vaccine to facilitate and increase vaccine presentation. Further, cultured dendritic cells can be used as a booster to prolong the effectiveness of methods that rely on the induction of an immune response, such as in photopheresis and vaccination protocols.

The present invention is further based on the observation that photochemical agents used in photopheresis are effective in photopheretic methods because they

increase the level of MHC Class I expression on the treated cells. Based on this observation the present invention provides methods for identifying agents for use in extracorporeal treatment methods in addition to the presently used photochemical agents. Further, this observation provides a means to optimize a treatment protocol by assaying for an increase in MHC Class I expression during agent treatments.

The methods and compositions of the present invention are used to treat a subject that has a disease that is mediated by or is conditioned upon the presence of circulating "disease effector" cells. Examples of disease effector cells include, but are not limited to, T cells, B cells, and/or infected white blood cells, such as virally or bacterially infected cells. Exemplary diseases that can be treated using the methods of the present invention include, but are not limited to, leukemia, lymphoma, autoimmune disease, graft versus host disease, and transplanted tissue rejection. In these conditions, an antigen that mediates the disease state (i.e., the "disease-associated antigen") is a peptide that is associated with (binds to) an MHC Class I site, an MHC Class II site or, to a heat shock protein that is involved in transporting peptides to/from MHC sites (i.e., a chaperone). Other conditions that can be treated using the present methods include condition in which a disease-associated antigen, such as a viral or bacterial peptide, is expressed on the surface of an infected white blood cell, usually in association with an MHC Class I or Class II molecule.

The methods and compositions of the present invention are useful for improving the effectiveness and specificity of therapeutic strategies that involve antigen presentation on any type of antigen presenting cell and in providing methods for inducing and augmenting an immune response to specific antigens. The invention is particularly useful for improving the efficacy of extracorporeal blood treatment methods, such as photopheresis, in subject populations for which photopheresis has proven of little or no value as a treatment modality, e.g., the 25% of subjects diagnosed with cutaneous T cell lymphoma for whom photopheresis has proven to be completely ineffective and the 50% of subjects in which the effect is transient and/or incomplete. The invention also provides a substantial cost savings by providing

methods and compositions that permit the generation of multiple therapeutic compositions for subsequent administration from a single procedure.

According to still another aspect of the invention, an antigen composition for enhancing a cellular immune system response is provided. The antigen composition contains disease-associated antigens that have been released from disease effector cells contained in blood and a detectable amount of a treatment agent, such as psoralen or other photoactivatable agent. The composition is formulated to contain an amount of disease-associated antigens for mixing with a single dose of dendritic cells to form a cellular vaccine.

According to yet another aspect of the invention, a process for producing a product for enhancing an immune response and the product produced thereby are disclosed. The process involves: (a) acidifying a preparation containing a plurality of disease effector cells for a period of time sufficient for the disease effector cells to release disease-associated antigens without lysing the cells; and (b) neutralizing the acidified preparation to form the product. In one embodiment of this method, beta2-microglobin is added to stabilize the prepared antigens.

The present invention further provides a titration point for determining when to stop agent treatment during the extracorporeal treatment and when to administer the treated blood to a subject. In general, these methods rely on art known methods to determine the course of MHC expression during the course of agent treatment of the extracorporeal blood. A treated blood mixture is ready for administration or combining with dendritic cells when an increase in MHC class I expression, as a result of the agent treatment, is observed/detected.

These and other aspects of the invention, as well as various advantages and utilities will be more apparent with reference to the detailed description of the preferred embodiments.

Brief Description of the Figures

Figure 1 is a graph that demonstrates the antitumor response of vaccinations against tumor growth using a therapeutic mixture comprising irradiated 2B4.11 tumor cells and dendritic cells.

Figure 2 shows the inhibition of tumor growth using dendritic cells (DAPC) alone.

Figure 3 shows the impact of 8-MOP/UVA treatment on MHC Class I expression.

Detailed Description of the Invention

A. General Description

The present invention provides improved methods for use in extracorporeal blood treatment for use in inducing an immune response in a subject. The improved methods use a synergistic combination of two therapeutic methods, extracorporeal treatment of blood with agents that increase MHC Class I expression (such as photopheresis) and dendritic or other antigen presenting cell-mediated immune therapy.

One embodiment of the present invention relates to the discovery that when combined, these two methods exert a synergistic therapeutic effect in treating a subject that is diagnosed as having a disease state that is mediated by circulating T cells, B cells or infected circulating cells, such as white blood cells infected by an infectious agent, such as, but not limited to, virus, bacteria, protozoa, etc.

B. Specific Descriptions

I) Extracorporeal Blood Treatment

According to one aspect of the present invention, an improved method for extracorporeal treatment of the blood of a diseased subject to induce an immune system response to one or more disease-associated antigens is provided.

In one embodiment, the methods of the invention comprise the steps of: 1) obtaining blood containing disease effector cells that express one or more disease associated antigens from the subject; 2) obtaining dendritic cells, or other antigen presenting cells, from the subject; 3) treating the blood containing the disease effector cells with an agent that increases MHC Class I expression (extracorporeal blood treatment); 4) introducing the dendritic cells, or other antigen presenting cells, into the treated blood mixture to form a therapeutic mixture; and (5) reinfusing or otherwise

introducing the therapeutic mixture into the diseased subject as a mixture of treated cells and dendritic cells or as a vaccine containing purified, antigen loaded dendritic cells.

The present invention further provides methods for identifying agents that can be used in the methods of the present invention as well as other extracorporeal blood treatment methods that are used to induce an immune response to a target antigen. Specifically, it has been found that the effectiveness of photopheresis is potentiated, in part, by the ability of the photochemical agent/treatment to induce MHC expression on the treated cells. The ability to increase MHC expression can be used as an assay point for identifying agents for use in the present method. Further, MHC expression can be used as a titration point for determining the optimum treatment agent/protocol for the present methods.

II) Disease Effector Cells and Conditions Treatable with the Present Methods

The methods of the present invention are useful for treating a diseased subject, i.e., a subject who has been diagnosed as having a disease that is mediated by "disease effector cells," "circulating aberrant cells," or "non-circulating disease effector cells," cells that express an antigen that is associated with a pathological condition, i.e. solid tumor cells.

As used herein, "disease effector cells" or "circulating aberrant cells" refers to cells that (1) are present in peripheral blood (preferably a T cell, a B cell, or a virally or bacterially infected white blood cell), (2) mediate a pathological or disease state and 3) express peptides or proteins that can be used to distinguish them from other similar cells that are not associated with the pathological condition. For example, a disease effector cell can be a T-cell that has undergone a transformation to be a tumor cell, a T-cell that mediates an autoimmune disorder or can be a cell infected with a virus, bacteria or other microorganism that results in the cell expressing one or more proteins/peptides of viral, bacterial, protozoan or microorganism origin. As used herein, "non-circulating disease effector cells" refers to cells that (1) are not present in peripheral blood (preferably a solid tumor or infected organ), (2) mediate a

pathological or disease state and 3) express peptides or proteins that can be used to distinguish them from other similar cells that are not associated with the pathological condition. For example, a non-circulating disease effector cell can be a solid tumor. Thus, disease effector cells (circulating and non-circulating) include, but are not limited to, malignant T cells, malignant B cells, T cells or B cells that mediate an autoimmune or transplanted tissue rejection response, virally or bacterially infected white blood cells or tissues that express viral or bacterial proteins/peptides and solid tumor cells. Preferably, the disease effector cells will express an antigen on the cell surface and will be T cells or B cells, more preferably T cells. The preferred disease effector cells are T cells belonging to a single clone. More preferably, the disease effector cells are T cells that are polyclonal or are tumor cells obtained from a solid tumor.

As described below, the agent treatment step of the methods of the present invention, for example photopheresis, damages the disease effector cells to the extent that the agent treated cells increase MHC Class I expression, and/or transport/release disease-associated antigens, but are not immediately lysed or killed. The transported/released peptides then are passed "baton-fashion" to the dendritic cell major histocompatibility complex molecules, either by entering empty MHC sites or by displacing peptides that are present in the added dendritic cell MHC Class I or Class II sites for presentation.

Thus, the methods of the invention are useful for treating diseases such as leukemias, lymphomas, solid tumors, metastatic tumors, autoimmune diseases, transplanted tissue rejection, and graft versus host disease. In addition, the methods of the present invention are useful for treating viral or bacterial conditions including HIV, malaria, etc. and other blood borne infections that are mediated by intracellular parasites or other factors, including, e.g., listeria, Epstein Barr virus, HTLV-1, herpes simplex, varicella, hepatitis A, B and C virus, protozoan, such as leishmania donovans. See, e.g., Goodman and Gilman's "The Pharmacological Basis of Therapeutics, W.A. Goodman Gilman *et al.*, Pergamon Press, N.Y., N.Y. (1990) for a description of protozoan infections which can be treated in accordance with the methods disclosed

herein. These infections include malaria, amebiasis, guardiasis, trichomoniasis, leishmaniasis, trypanosomiasis, and toxoplasmosis.

III) Subject

The methods of the present invention are intended to be used in treating any mammalian subject, so long as the subject is in need of inducing an immune response to disease effector cells. The methods of the present invention are preferably used to treat humans.

Because modulation of the cellular immune response is important to the execution of the invention, it is preferred that the recipient subject of the methods of the present invention have a competent immune system, as evidenced by, for example, near normal absolute levels of CD8 positive T cells.

IV) Blood removal or Tissue Removal

The first step used in practicing one aspect of the present invention is to remove blood from a subject that contains disease effector cells as defined above or tissue that contains the non-circulating disease effector cells. A variety of methods are known in the art for removing blood containing disease effector cells, for isolating disease effector cells for removed blood, for example by using centrifugation, and for removing non-circulating cells, such as solid tumors. Some of these methods are described in detail below. A skilled artisan can readily adapt any of the blood removal/tissue removal, separation and culturing methods known in the art for use in the present methods to obtain blood containing disease effector cells, a population of disease effector cells that express one or more target antigens, or a population of non-circulating disease effector cells.

The amount of blood or tissue removed will be based primarily on the disorder being treated and the therapeutic protocol that is employed. For example, for treating CTCL using a therapeutic protocol calling for from a single injection, to weekly or monthly injection injections of treated cells, approximately from about 200 cc to about 750 cc of blood is removed. A skilled artisan can readily determine the amount of blood that needs to be removed for treatment based on the number of disease effector

cells per cc of blood. As can be readily appreciated, the blood used in the extracorporeal treatment may be removed over a course of several sessions. Alternatively the blood can be removed, treated and re-infused in a continuous process. For treating a solid tumor, as much of the tumor is removed for both therapeutic purposes as well as to provide a source of antigen for dendritic cell loading.

V) Agent Treatment of the Disease Effector Cells

After removal of blood from the subject, the blood containing disease effector cells, or purified disease effector cells, is subjected to extracorporeal treatment using a treatment agent. As used herein, "extracorporeal blood treatment" refers to the process in which the blood of a diseased subject is removed and is treated with an agent to form an agent-treated blood sample.

One observation of the present invention is that photochemical agents that have been used in photopheretic methods cause a previously known increase in MHC expression, particularly MHC Class I, on the treated cells. Accordingly, the agent used in the present method can be any agent that will act to increase MHC expression, particularly MHC Class I expression. For cells that do not express MHC proteins, any agent that leads to an increase in cellular proteolysis can be used. The treatment agent may further be an agent that has an affinity for an important component of blood cells or for a particular disease effector cell.

The agent used in the present method can be, but is not limited to, chemical agents and physical agents. For example, the agent may be a chemical compound that induces MHC expression and/or cellular proteolysis, such as a photoactivatable drug such as psoralen. Alternatively, the agent may be a physical treatment that the blood is subjected to. For example, UV light, heat shock and other environmental stresses have been shown to induce MHC Class I expression in other experimental contexts. As outlined below, a skilled artisan can readily identify agents for use in the present methods based on the ability of the agent to induce MHC expression on treated (disease effector) cells.

Exemplary photoactivatable chemical agents that can be used with the present methods include, but are not limited to, psoralens, porphyrins, pyrenes, phthalocyanine,

photoactivated cortisone, photoactivated antibodies specifically reactive with the disease effector cells present in the blood, photoactivatable dyes, and monoclonal antibodies which have been linked to porphyrin molecules. Exemplary non-photoactivated chemical agents include, but are not limited to, chemotherapeutic agents, such as cyclophosphamide or methotrexate, and cytokines, such as TNF-alpha, and interferon-gamma. Exemplary non-chemical agents include, but are not limited to, UVA iradiation, X-ray irradiation, gamma-ray iradiation, hydrostatic or other pressure, heat or cold shock and ultrasound.

The psoralens are a preferred class of photoactivatable agent that are used in current photopheretic methods. Following oral administration, psoralens are absorbed from the digestive tract, reaching peak levels in the blood and other tissues in one to four hours and are excreted almost entirely within 24 hours following oral administration. These agents can alternatively or additionally be added directly to the extracorporeal bloodstream.

The psoralen molecules are inert prior to exposure to irradiation and are transiently activated to an excited state following irradiation. The transiently activated psoralen molecules are capable of forming photoaddition products with cellular DNA, proteins, or lipids and generating other reactive species, such as singlet oxygen, which are capable of modifying other cellular components, e.g., cell membrane and cytoplasmic components such as proteins and aromatic amino acids. Although other agents such as mitomycin C and cis-platinum compounds also damage DNA by cross-linking strands of the nucleic acid, such alternative agents remain in an active state following reinfusion, cause systemic adverse effects and thus are not as desirable as psoralens for achieving the purposes of the invention.

The preferred psoralens include 8-methoxypsoralen (8-MOP), 4' aminomethyl-4,5', 8 trimethylpsoralen (AMT), 5-methoxypsoralen (5-MOP) and trimethyl psoralen (TMP). These and other analogs of 8-MOP are described in Berger, *et al.*, *Annals N.Y. Acad. Science* 453:80-90 (1985). The conditions for oral administration of 8-MOP are described in U.S. Patent No. 5,147,289. 8-MOP is the preferred psoralen for use in accordance with the methods of the invention.

When using a photoactivatable agent, the agent-treated blood sample is further irradiated with ultraviolet or visible light during the agent treatment step, for example see U.S. Patent No. 5,462,733, issued to Edelson *et al.*, for a discussion of the irradiation conditions for activating photoactivatable agents such as psoralen compounds. Photopheresis procedures also are described in U.S. Patent Nos. 4,321,919; 4,398,906; 4,428,744; 4,464,166; and 5,147,289, all issued to Edelson *et al.*

The treatment of the blood or purified disease effector cells with the treatment agent can be done, as is known in the art, on a continuous stream of blood or in a batch wise manner. Continuous extracorporeal treatment can be divided into five stages: (1) blood collection; (2) centrifugation; (3) agent treatment; (4) cell pooling and (5) reinfusion. The choice of the agent treatment method used will be based primarily on the disorder being treated, the agent used and the facilities that are available.

During the agent treatment step, such as in the use of a photoactivatable agent, the agent can be present within or on the surface of the cells of the blood sample. This is typically accomplished by administering the agent to the subject prior to obtaining the blood for extracorporeal treatment or by injecting the agent directly into the extracorporeal blood stream when using a continuous stream treatment method.

In contrast to the literature that suggests that an underlying mechanism of photopheresis involves subtly modifying antigen presenting cells to enhance their ability to induce an immune system response, as shown in the Examples, treatment of the extracorporeal blood leads to an increase in MHC Class I expression and allows for an increase in the rate and extent that antigens are transported and bound (weakly) to surface MHC molecules. Such antigens then become available for presentation by dendritic or other antigen presenting cells, which are added to the blood sometime prior to reinfusion.

VI. Dendritic Cells Addition

The extracorporeal treatment methods of the present invention rely on the use of dendritic cells, or other antigen presenting cells, in combination with the

extracorporeal agent treatment. Dendritic cells, or other antigen presenting cells, are added directly or indirectly to the blood containing the disease effector cells to form a therapeutic mixture comprising agent treated disease effector cells and added dendritic cells prior to re-infusion into the subject.

Dendritic cells are added to the blood or purified disease effector cells (before or after agent treatment) in an amount sufficient to enhance the immune system response of the subject to the one or more disease-associated antigens. Such an increase is measured relative to the level of immune system response that would have been induced had the extracorporeal blood treatment been performed in the absence of the added dendritic or antigen presenting cells. In general, the amount of dendritic cells contained in a single dose to achieve this purpose is approximately 1 million cells, but can range from about one thousand cells to about one hundred million cells per dose. However, as described below, larger numbers of dendritic cells can be prepared and introduced into the treated blood to obtain multiple doses of antigen-loaded and non-antigen loaded dendritic cells. In general, these cell numbers are consistent with the cell numbers described in Zitvogel, L., *et al.*, *J Exp Med* 184:87-97 (1996) for an animal model in which peptide-loaded dendritic cells were administered to a tumor-challenged mouse to enhance the animal's specific immune system response to a solid tumor. According to Zitvogel, *et al.*, in a weakly immunogenic tumor model, animals were injected three to four times, starting at day 4 or day 8 after tumor establishment and subsequently, every 4 days, with 3-5 x 10^5 dendritic cells pulsed with peptides. In the more immunogenic tumor model, animals were injected on days 14, 21, and 28 after initial intradermal (i.d). tumor inoculation. In an analogous manner, booster immunizations for human subjects are designed to take into consideration the immunological state of the subject in accordance with standard clinical practice.

Methods for obtaining dendritic cells are well known in the art and are described in detail below. In general, the dendritic cells used will be obtained from the subject sometime prior to reinfusion of the treated blood. The dendritic cells can be obtained at the same time as the blood is removed for treatment or can be obtained prior to of after blood removal.

In one application, the dendritic cells can be activated *in vivo* prior to their removal from the subject. A variety of method can be used to activate dendritic cells *in vivo* prior to their removal. For example, activation can be accomplished by administering a sufficient dosage of GM-CSF to the subject prior to removing of the dendritic cells. As used herein a "sufficient dosage of GM-CSF" is the amount and frequency of administration of GM-CSF that is sufficient to increase the number and/or activation state of the dendritic cells in the subject. Exemplary dosages of GM-CSF for increasing the number and/or activation state of the subject's dendritic cells are provided in the Examples (see, "Isolation of Dendritic Cells from Human Blood").

In such a use, the steps of the present method comprise: 1) administering GM-CSF to the subject prior to removal of dendritic cells, wherein the dosage of GM-CSF is sufficient to increase the number and/or activation state of the dendritic cells in the subject; 2) obtaining blood containing disease effector cells that express one or more disease associated antigens from the subject; 3) obtaining and culturing, *in vitro*, dendritic cells, or other antigen presenting cells, from the subject; 4) treating the blood containing the disease effector cells with an agent that increases MHC Class I expression (extracorporeal blood treatment); 5) introducing the dendritic or other antigen presenting cells into the treated blood mixture to form a therapeutic mixture; and (6) reinfusing or otherwise introducing the therapeutic mixture into the diseased subject.

In general, the isolated dendritic cells can be introduced at any stage during the extracorporeal treatment process: (1) during the blood or tissue collection step; (2) prior to or after the disease effector cell isolation steps (i.e. centrifugation); (3) prior to or after the agent treatment step; and (4) prior to or after disease effector cell pooling. Thus, the dendritic cells can be introduced to the treated blood/tissue before, during, or after agent treatment. The advantage of introducing the dendritic cells during the blood collection stage is that a high dendritic cell concentration can be achieved by adding the dendritic cells directly to the blood collection bag. However, if centrifugation procedures are used to separate blood into plasma, white blood cell and red blood cell components it is not 100% efficient and some dendritic cells may enter

the plasma and/or red blood cell fractions and not be proximal to the antigens immediately upon their release from the agent treated cells in the white blood cell fraction. Accordingly, it is preferred that the dendritic cells be introduced into the extracorporeal blood stream during one or more of the agent treatment stages to ensure sufficient numbers of peptide-loaded dendritic cells for subsequent reinfusion (e.g., injection).

It is believed that centrifugation forces, alone, or in combination with agent treatment, such as during irradiation in the presence of a photoactivatable agent, facilitates the release and transfer of disease-associated antigens from the disease effector cells by increasing contact between the dendritic cells and the disease effector cells. Accordingly, introducing the dendritic cells during centrifugation advantageously places the released disease-associated antigens in close proximity to the added dendritic cells, thereby facilitating transfer of the released peptides from the disease effector cells to the MHC sites of the dendritic cells and, presumably, minimizing enzymatic digestion of the released peptides.

Alternatively (or additionally), the dendritic cells can be introduced into the treated blood/tissue during the agent treatment stage. For example, the agent treatment stage of photopheresis is performed by passing the disease effector cell containing fraction through an irradiation exposure field that is positioned between opposing irradiation sources. Introducing the dendritic cells during the irradiation stage places the dendritic cells in close proximity to the disease effector cells at the time of their irradiation, thereby facilitating transfer of the released peptides to the dendritic cells and minimizing enzymatic digestion.

Further, the dendritic cells can be added prior to agent treatment and are then treated along with the disease effector cells. Although not intending to be bound to a particular theory, it is believed that agent treatment of the dendritic cells (i.e., the photopheretic irradiation of the dendritic cells in the presence of a photoactivatable agent) may also activate the dendritic cells to release cytokines (e.g., IL-12, IFN-gamma, TNF-alpha, GM-CSF, IL-3), an effect that further enhances the immune

system response following reinfusion of the antigen-loaded dendritic cells to the subject.

Alternatively (or additionally), the dendritic or other antigen presenting cells can be added to the blood administration bag prior to reinfusing the agent treated disease effector cells. Methods for introducing the dendritic or other cells at any stage in the treatment process are based on conventional procedures and can be readily adapted for adding dendritic cells into a treated blood preparation. For example, at each stage of treatment process, conventional intravenous tubing connections provide access ports through which the dendritic cells can be injected into, for example, the blood collection bag, the centrifugation apparatus, the tubing located in the agent/irradiation treatment chamber and the blood re-infusion bag. Additional reagents, such as cytokines, also can be introduced via these same infusion ports.

In the above methods, the dendritic or other antigen presenting cells can be added directly or indirectly to the blood or tissue. As used herein, direct addition refers to adding the dendritic cells directly to the blood or tissues (before or after treatment) under condition in which there can be direct cell-to-cell contact between the added dendritic cells and the disease effector cells.

As used herein, indirect addition refers to adding dendritic cells to the blood or tissues (before or after treatment) such that the dendritic cells do not come into direct contact with the disease effector cells. For example, a filter membrane, dialysis membrane or other partitioning membrane can be placed in between the dendritic cells and the disease effector cells. Such a partition acts to allow the transfer of disease associated antigens to the dendritic cells but does not allow mixing of the cell types. The preferred partitions will have a pour size of no greater than about 6 microns since this is the approximate size of the small cell that would need to be prevented from passing through the partition. There is no lower limit, however, the pour size must be large enough to allow passage of antigens and other cytokines released from the treated disease effector cells. Alternatively, the treated cells can be remove from the treatment solution, for example by centrifugation, leaving released diseased associated antigens, and the dendritic cells can be added to the resulting cell free solution.

Indirect addition is preferred in most methods because it avoids potential problems that may be associated with reintroduction of treated disease effector cells to a subject.

The dendritic cells preferably are obtained from peripheral blood but may be obtained from bone marrow, lymph nodes, infiltrated tumors and/or rejected organs. The dendritic cells should be "genetically identical" to the cells of the subject. Accordingly, the dendritic cells of the invention are preferably autologous cells: being obtained from the subject or an identical twin of the subject, or are genetically engineered to be recognized as an autologous cell by the subject's immune system.

Theoretically it is possible to isolate large numbers of dendritic cells from peripheral blood (e.g., by an affinity method in which the dendritic cells are specifically absorbed from the blood and concentrated), for example see Radmayer *et al., Int. J. Cancer* 63:627-632 (1995). However, it is preferred that the dendritic cells be cultured *in vitro* to expand their number prior to introducing the cells to the treated extracorporeal blood stream. In general, the cultured dendritic cells have the same or greater presentation characteristics (i.e., the number and type of MHC molecules) on their surfaces a naturally-occurring dendritic cells that have been freshly isolated from the subject.

The dendritic cells can be altered, for example, by increasing the number of empty Class I or Class I sites on the surface of the cell, prior to introducing the cells to the treated blood. This may be accomplished in accordance with the methods disclosed in PCT application number US93/11220, publication number WO 94/11016, entitled "Specific Immune System Modulation" (Edelson, R. *et al).* In addition, culturing dendritic cells provides a source of dendritic cells that can be used for booster inoculations.

Numerous references have described culturing dendritic cells, contacting the cultured dendritic cells with a purified antigen to form an antigen-loaded dendritic cell and administering the antigen-loaded dendritic cell to an animal to enhance a specific immune response to the antigen. For example, Steinman *et al.* (PCT/US93/03141, publication no. WO 93/20185) disclose a method for producing proliferating cultures of dendritic antigen presenting cell (DAPC) precursors. The method involves isolating

the precursors and culturing the precursors in the presence of a cytokine. Steinman *et al.* report that GM-CSF is an essential cytokine for dendritic cell culturing *in vitro*. Accordingly, Steinman *et al.* recommend administration of GM-CSF to a subject prior to sampling the subject's blood to obtain dendritic cell precursors for proliferation *in vitro*. Steinman *et al.* further report that the cultured, immature dendritic cells can be pulsed with antigen *in vitro* and will phagocytose the antigen and process it into a form which is presented on the dendritic cell surface, i.e., the Steinman dendritic cells must phagocytose the antigen for proper antigen presentation in Class II.

The Steinman method involves (a) providing a tissue source (e.g., blood, bone marrow) containing dendritic cell precursors; (b) treating the tissue source to increase its proportion of dendritic cell precursors to obtain a population of cells which is suitable for culture *in vitro* (e.g., by contacting the tissue source with GM-CSF); (c) culturing the tissue source on a substrate and in a culture media containing GM-CSF, or a biologically active derivative of GM-CSF, to obtain proliferating nonadherent cells and cell clusters; (d) subculturing the nonadherent cells and cell cultures to produce cell aggregates comprising proliferating dendritic cell precursors; and (e) serially subculturing the cell aggregates one or more times to enrich the proportion of dendritic cell precursors.

Mature dendritic cells are produced from the proliferating cell cultures by continuing to culture the dendritic cell precursors for a period of time sufficient to allow these cells to mature into mature dendritic cells. Mature dendritic cells are identified by cell markers such as, for example, high MHC Class II, 2A1 positive granules, and interdigitating cell (NLDC) antigen. In contrast to the Steinman *et al.* teachings, a preferred embodiment of the instant invention involves introducing mature dendritic cells into the treated blood to form disease-associated antigen-loaded dendritic cells. Thus, there is no requirements that the dendritic cells of the instant invention be in an immature state and capable of phagocytosis to present the disease-associated antigens of the invention.

Dendritic cells (or precursors) are cultured in the presence of GM-CSF, IL-4 and fibroblast growth factor at a concentration that is sufficient to promote the survival

and proliferation of the dendritic cell precursors. This amount depends on the amount of competition from other cells (e.g., macrophages ad granulocytes) for the GM-CSF, etc., as well as on the presence of GM-CSF, etc., inactivators in the cell population. In general, dendritic cells are cultured in the presence of between about 1 and 1,000 U/ml of GM-CSF. More preferably, dendritic cells that are obtained from blood are cultured in the presence of GM-CSF at a concentration of between about 30 and 800 U/ml. Most preferably, the GM-CSF concentration is between about 400-800 U/ml for culturing proliferating human dendritic cells from blood. Higher concentrations of GM-CSF (e.g., between 500-1,000 U/ml) are preferred for culturing dendritic cells obtained from bone marrow. The GM-CSF may be isolated from natural sources, produced using the recombinant DNA techniques or prepared by chemical synthesis. "GM-CSF" is defined as any bioactive analog, fragment or derivative of the naturally occurring (native) GM-CSF. This definition includes fragments and derivatives of GM-CSF provided that the fragments or derivatives promote the proliferation and culture of dendritic cell precursors and, in addition, can be identified by their ability to bind to GM-CSF receptors on the appropriate cell types.

Additional cytokines may be optionally included in the culture medium to further increase the yield of dendritic cells. These include such cytokines as IL-4 (at approximately the same U/ml as GM-CSF (See, e.g., L. Zitvogel, *et al.*, *J Exp Med* 183:87-97 (1996)); IL-1 alpha and beta (1-100 LAF U/l); TNF-α (5-500 U/m); IL-3 (25-500 U/ml); monocyte-macrophage colony-stimulating factor (M-CSF, 100-1,000 U/ml); granulocyte colony-stimulating factor (G-CSF, 25-300 U/ml); stem cell factor (SINGLE-CHAIN FORMS, 10-100 ng/ml); IL-6 (10-100 ng/ml); and FGF (1ng-500 U/ml). TNFα at concentrations from about 10-50 U/ml reportedly increases dendritic cell yields several fold. A skilled artisan can readily adapt know dendritic cell culturing methods for using with the present invention.

A panel of monoclonal antibodies may be used to identify and characterize the cells in the GM-CSF-expanded cultures to ensure that they are dendritic or other antigen presenting cells. Antibodies that are suitable for identifying mature dendritic cells include, but are not limited to: (1) those which bind to the MHC Class I antigen

(M1/42 anti-MHC Class I, ATCC number TIB 126); (2) those which bind to the MHC Class II antigen, B21-2 anti-MHC Class II, ATCC number TIB 229), (M5/114 anti-MHC Class II, ATCC number TIB 120); (3) those which bind to heat-stable antigen (M1/69 anti-heat stable antigen, HSA, ATCC number TIB 125); (4) 33D1 anti-dendritic cell antibodies, ATCC number TIB 227; (5) those which bind to the interdigitating cell antigen (NLDC 145 anti-interdigitating cell, Kraal, G., *et al., J Exp Med* 163:981 (1986)); and (6) those which bind to antigens in granules in the perinuclear region of mature dendritic cells (monoclonal antibodies 2A1 and M342, Agger, R., *et al., Int Rev Immunol* 6:89 (1990)). Additional antigens that are expressed by dendritic cells that can be used to identify mature dendritic cells include CD44 (identified with monoclonal antibody 2D2C), and CD11b (identified with monoclonal antibody M1/70). (See, e.g., Monoclonal Antibodies, New York, Plenum 1980, Ed. R. Kennett *et al.*, pp. 185-217 for a description of some of the monoclonal antibodies which are used to identify antigens which are expressed on mature dendritic cells). One skilled in the art will recognize that other antibodies may be used to characterize and identify mature dendritic cells and also to characterize and identify precursor dendritic cells and to distinguish these stages of dendritic cell growth.

Although mature dendritic cells are preferred for introduction into the treated blood, either during or following agent treatment, immature dendritic cells also can be pulsed with the disease-associated antigen preparations of the invention. Thus, contacting the mature or immature dendritic cells *in vitro* with the antigen preparations of the instant invention results in a composition containing antigen-loaded dendritic cells in which the antigen is presented on the surface of the dendritic cells. Although not intending to be bound to a particular theory, it is believed that the mature dendritic cells present antigen by loading the (released, disease-associated) antigen directly into the empty MHC sites or, alternatively, by exchanging the disease-associated antigens for peptides that already are present in the MHC sites of the mature dendritic cells. In contrast, the immature dendritic cells present antigen by the foregoing mechanism, as well as by phagocytosing released antigens, processing the released antigens into

smaller fragments, and expressing the smaller fragments in association with MHC molecules on the surface of the antigen presenting cells.

Although various references have disclosed culturing dendritic cells and the use of such cultured cells in modifying an immune system response, the introduction of an exogenous antigen presenting cell, such as a dendritic cell, particularly a mature dendritic cell, into an extracorporeally treated blood stream to enhance an immune system response has not been previously described. Further, the art did not teach that antigen loaded or non-antigen loaded dendritic cells can be used for booster inocluations following reinfusion of extracorporeally treated blood. It is believed that the absence of such a teaching within the art is consistent with the lack of a complete understanding of the mechanism underlying extracorporeal blood treatment methods such as photopheresis, i.e., the failure by those skilled in the art to recognize that photopheresis induces release of disease-associated peptides. In the absence of recognition of this effect of photopheresis, one would not be motivated to add exogenous antigen presenting cells to the treated blood for the purpose of enhancing an immune response to the released disease-associated antigens.

In addition to dendritic cells, other types of antigen presenting cells can be used in accordance with the methods of the invention, i.e., by substituting these alternative antigen presenting cells for the dendritic cells in the methods and compositions disclosed herein. For example, Canadian patent application 2,069,541, entitled "Induction of an antigen-specific T-lymphocyte response" (inventors, Melief, *et al).* describes antigen presenting cells which are incapable of loading peptide into MHC sites, i.e., the cells have an antigen processing defect which prevents proper antigen presentation at the cell surface. As a result, the MHC sites of these defective cells are empty and available for binding to the disease-associated antigens of the instant invention. In general, these cells have a defect in one of the cellular gene products responsible for peptide transport into the subcellular compartments (endoplasmic reticulum and golgi apparatus) where peptide loading into MHC Class I or MHC Class II molecules takes place. The exemplary processing defective cell lines include RMA-S cells of murine origin and 174.CEM T2 cells of human origin (Salter, R.D. *et*

al., *EMBO J* 5:943-949 (1986)). There is no requirement that a processing defect in the antigen presenting cells of the invention be complete, provided that the cells express an increased population of cell surface MHC Class I or Class II molecules which are devoid of endogenously processed peptides. Such cells are capable of inducing a primary CTL response when appropriately loaded with MHC Class I binding peptides.

The antigen presenting cells used in the present methods can also be antigen presenting cells that have been treated with antisense oligonucleotides to inactivate one or more genes responsible for proper antigen processing and presentation at the cell surface. This approach increases the number of empty Class I molecules on the antigen presenting cells, thereby making these cells more capable of binding antigenic peptides released from treated disease effector cells. Thus, for example, dendritic cells or other antigen presenting cells are incubated with antisense oligonucleotides under conditions to permit hybridization of the antisense oligonucleotide to the processing gene or mRNA (e.g., the human TAP-2 gene). The TAP genes encode proteins which are necessary for the transport of relevant cytoplasmic peptides to Class I molecules, prior to their joint transport to the cell surface. Therefore, inhibition of the formation of TAP proteins, diminishes filling of Class I molecules with peptides. This circumstance will, hence, increase the amount of surface "empty" Class I. Exemplary conditions and oligonucleotides for inactivating the TAP-2 gene in cultured RMA and EL4 cells or freshly isolated splenocytes are provided in Nair, S. *et al.*, *J Immunology* 156:1772-1780 (1996). In particular, S. Nair report that MHC Class I expression was decreased in approximately 30% of the cells which had been treated with the AS-1 or AS-2 antisense oligonucleotides. These oligonucleotides are complementary to two different regions of the TAP-2 mRNA and were synthesized as 25 nucleotide long phosphorothioate derivatives. (See, Nair, S. *et al.*, *ibid.* for sequence and storage information for these antisense oligonucleotides).

The following procedure, based upon that described by Nair, S. *et al.*, *ibid.*, is used for preparing a preferred antigen presenting cell for use in the present method. Briefly, antigen presenting cells (preferably in log phase) are washed in medium (e.g.,

Opti-MEM medium, Life Technologies), resuspended in medium at 5 to 10 x 10^6 cells/ml and added to 24-well or 6-well plates. A cationic lipid, e.g., Lipofectin (Life Technologies), is used to deliver the antisense oligonucleotides into cells as described by Chiang *et al.*, *J Biol Chem* 266:18162 (1991). Other methods known in the art for delivering oligonucleotides into cells (e.g., receptor-mediated delivery, electroporation) can be substituted for the Lipofectin method described herein. The oligonucleotides and Lipofectin are added to medium at the desired concentration (see below) and mixed in a 12 x 75 mm polystyrene tube at room temperature for 20 min. The complex is added to the cells to achieve a final concentration of 400 nM oligonucleotide and 15 µg/ml Lipofectin and incubated at 37°C for 6 to 8 hours. The antisense-treated cells are washed, incubated at subphysiologic temperature (preferably in the range 23 to 30°C) for 24 to 48 hours, and analyzed for MHC Class I expression by flow cytometry, used as stimulators for CTL induction using standard procedures (e.g., chromium release assay) and/or used as antigen presenting cells for subsequent presentation *in vivo* of disease-associated peptides (e.g., by reinfusing or otherwise introducing the antisense-treated cells to the extracorporeal blood system or by incubating the antisense-treated cells *in vitro* with disease-associated peptides and subsequently introducing the peptide-loaded antisense-treated cells to the subject. Preferably, the antisense-treated cells are incubated with beta2-microglobulin prior to, or concurrent with, incubating the antisense-treated cells with disease-associated peptides. It is believed that prior treatment or co-incubation of the antisense-treated cells with beta2-microglobulin and disease-associated peptide facilitates loading of the peptides into the empty MHC Class I sites of the antisense-treated antigen presenting cells.

In a particularly preferred embodiment, one or more cDNAs (or gene sequences) encoding the following proteins are introduced (e.g., transfected) into a processing defective cell line (e.g., a T2 cell line) to obtain an improved antigen presenting cell for use in accordance with the methods of the invention: (1) a cytokine(s) (e.g., GM-CSF, IL-12); (2) an accessory molecule such as a costimulatory molecule(s) (e.g., B7-1/CD80 and B7-2/CD86 (Mayordomo, J., *et al.*, *Nature Med*

1:1297-1302 (1995)) or an adhesion molecule (e.g., ICAM-1/CD54; ICA-3/CD50 (Young, J. *et al. J Exp Med* 183:7-11 (1996) and references cited therein); and (3) one or more of the MHC Class I molecules of the subject. Human B7-1 and B7-2 are described in Freedman, A.S., *et al.*, *J Immunol* 137:3260-3267 (1987), Freeman, G.J., *et al.*, *J Immunol* 143:2714-2722 (1989), Freeman, G.J., *et al.*, *Science* 262:909-911 (1993) and Azuma, M., *et al.*, *Nature* 366:76-79 (1993). Thus, an improved dendritic stock cell line can be prepared by introducing one or more cDNAs encoding a cytokine (preferably, GM-CSF) and an accessory molecule (preferably, a B7 and/or ICAM-1 molecule) into a processing defective cell line (preferably, a T2 cell line). (See, for example, Paglia, P., *et al.*, *J Exp Med* 183:317-322 (1996), for an exemplary procedure for transducing dendritic cells with the gene encoding GM-CSF in a mouse model system). The improved dendritic stock cell line can be prepared and maintained in accordance with standard procedures known in the art for introducing and expressing genetic material in mammalian (preferably, human) cells. Moreover, in the preferred embodiments, the dendritic stock cell line is used to prepare subject specific antigen presenting cells by, for example, introducing the cDNA encoding the subject's MHC Class I molecules into the stock cell line using standard genetic engineering procedures. Of course, such transformed antigen presenting cells are treated (e.g., gamma-irradiated) to prevent further cell division prior to administration to the subject. The amount and nature of irradiation that is sufficient to prevent further cell division is determined empirically by irradiating the cells with a preselected radiation source (e.g., gamma-irradiation, 8-MOP and ultraviolet irradiation, X-irradiation, 8-MOP and visible irradiation) over a range of intensities (e.g., 1000 to 3000 rads) for preselected time periods (e.g., 0.5 minutes to 24 hours) and observing whether any clones develop over a period of time, usually a one month period. The amount and nature of the irradiation is selected which is sufficient to prevent any clones from developing during this time period. Typically, 2000 rads of gamma-irradiation reportedly is sufficient to achieve this purpose. (See, e.g., Zitvogel, L., *et al.*, *J Exp Med* 183:87-97 (1996)).

In contrast to the above-described transformed cell lines which must be irradiated to prevent further cell division in vivo, cultured dendritic cells that are

derived from the subject do not require irradiation prior to reintroduction to the subject. However, dendritic cells which have been loaded with disease-associated antigens preferably are irradiated (e.g., with gamma-irradiation or within the extracorporeal stream during photopheresis) prior to reintroduction (e.g., i.v. infusion) to the subject to prevent in vivo processing and undesired presentation by the dendritic cells of autologous (non-disease-associated) peptides (e.g., peptides which could mediate an autoimmune response) following administration to the subject.

GM-CSF and IL-4, cytokines that are important for dendritic cell culturing, optionally are coadministered with the antigen-loaded dendritic cells to the subject to further enhance the subject's immune response to the presented antigen. In addition to these key cytokines, TNF-α and IL-12 also reportedly are important for dendritic cell mediated immune system modulation, presumably by inducing a CD8 T cell response. Accordingly, the methods of the invention optionally include the step of introducing the dendritic cells to the treated blood in the presence of one or more of the following cytokines, GM-CSF, IL-4, TNF-α and IL-12. Preferably, a TNF-α and/or IL-12 are introduced with the dendritic cells to the treated blood or at some stage prior to reinfusion.

At any stage in the extracorporeal treatment process, following introduction of the dendritic cells, a sample of the therapeutic mixture may be taken and assayed to determine, for example, the number of viable dendritic cells and/or the number of disease-associated antigen-loaded dendritic cells, using the markers listed above. By using these markers in conjunction with fluorescein diacetate, which cytoplasmically labels non-viable cells, the number of viable dendritic antigen presenting cells can be determined. The number of viable dendritic cells can be determined in accordance with standard practice, e.g., by trypan blue exclusion assay. The number of antigen-loaded dendritic cells can be determined, for example, using a cytotoxic T cell assay as described in PCT publications WO 94/02156 or WO 94/21287. Alternative procedures for assessing the viability and/or functional activity of the dendritic cells are known to those of ordinary skills in the art and can be performed using routine experimentation. See, e.g., PCT publications WO 93/20185, WO 93/03766,

WO 95/29698, WO 94/02156, WO 94/20127, WO 91/13632, WO 95/28479, WO 94/21287 and CA patent application 2,069,541.

VII. Reinfusion

After the dendritic or other antigen presenting cells are added to the treated blood (directly or indirectly) to form a therapeutic mixture, the mixture can be re-infused into the subject as a treated dendritic cell/treated cell mixture or can be further processed to obtain isolated, antigen-loaded dendritic cells. The former provides an efficient method that can further provide an additional source of disease cell antigen (i.e. the agent treated disease cell antigens), while the later removes potentially viable disease effector cells.

Re-isolating the dendritic cells prior to reinfusion provides a means for obtaining multiple re-infusion doses and a source of antigen-loaded dendritic cells that can be used for immunotherapy. Specifically, the peptide-loaded dendritic cells generated by contacting dendritic cells with agent treated blood, can be used as an immunogen by administering the cells to a subject in accordance with methods known in the art for eliciting an immune response. Preferably, the dendritic cells are injected into the same individual from whom the source cells were obtained. The injection site may be subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.m.), intradermal (i.d.), or intravenous (i.v.). Intravenous administration of the antigen-loaded dendritic cell is the preferred route of administration.

The number of antigen-loaded dendritic cells that are administered to the subject varies as a function of the antigen, the immune status of the subject the size of the subject and the disease that is treated. In a preferred embodiment, blood is used as the tissue source and preferably, the subject is first treated with cytokine to stimulate hematopoiesis. Following isolation and expansion of the dendritic precursor cells, the precursors are contacted with the antigen preparations made from agent treated disease effector cells and/or alternatively are stimulated by cytokines (e.g., GM-CSF) to maturity before introducing the antigen. The antigen-loaded dendritic cells are reintroduced to the subject in sufficient quantity to invoke an immune response. In general, between $1x10^6$ and $10x10^6$ dendritic cells constitute a single dose for injection

into the subject. Preferably, between about 1 to 100 micrograms of antigen in its presented form, is administered per dose.

For example, to prepare a sufficient number of dendritic cells (with loaded antigen) for between ten and one-hundred doses, from about $1x10^8$ to about $1x10^9$ dendritic cells are introduced into the extracorporeal blood sometime prior to, during or following agent treatment. Following contact with the disease-associated antigens contained in the extracorporeal treated blood, the dendritic cells either process (phagocytose) the disease-associated antigens and present the processed antigen in association with MHC Class I or II molecules or directly load the disease-associated antigens into MHC Class I or II molecules. Eventually, these antigen-loaded dendritic cells are pooled during the final stage of the extracorporeal treatment and prior to re-infusion. The pooled dendritic cells, with or without the treated disease effector cells can be stored for subsequent booster immunizations. Preferably, these pooled antigen-loaded dendritic cells and other treated disease effector cells are distributed in aliquots prior to storage, each aliquot containing an amount of dendritic cells sufficient for a single dose for injection into the subject. The cells can be stored in accordance with standard methods to retain cell viability. Preferably, the cells are stored at -70°C.

VIII) Non-Antigen Loaded Dendritic Cells

In another embodiment of the present invention, it was found that non-antigen loaded dendritic cells can be used for one or more booster inoculations for subjects that are being treated by the methods of the present invention, are being treated by other methods in which it is desired to elicit an immune response (for example during routine vaccination protocols) or are in need of an increase in immune activity (for example to inhibit tumor cell growth). Specifically, isolated or cultured dendritic cells that have not been contacted with treated disease effector cells, can be introduced into a subject as a means for increasing cellular and humoral immune responses. In an application of this methods, the dendritic cells are treated with an agent in a manner analogous to the treatment described for the disease effector cells. As shown in the Examples, dendritic cells that are treated with a photochemical agent, greatly reduced

or eliminated tumor growth in an animal model without needing to contact the dendritic cell with a treated disease effector cell or disease associated antigen..

IX Antigen Containing Compositions

Although the prior art reports antigen-loaded dendritic cells for enhancing cellular immunity to a mixture of solid tumor antigens or to purified peptide antigens, methods for preparing disease-associated antigens by subjecting blood to agent treatment (such as photopheresis) and using the treated blood as an antigen source have not been described. Thus, according to another aspect of the invention, antigen compositions for enhancing an immune system response are disclosed. The antigen compositions have in common a plurality of disease-associated antigens that have been released from disease effector cells contained in blood by extracorporeal agent treatment as herein described. In addition, the compositions optionally contain a detectable amount of one or more protease or peptidase inhibitors. Exemplary protease or peptidase inhibitors and compositions containing the same include: (1) a mixture containing bestatin (30 uM), thiorphan (10 uM) and captopril (10 uM); (2) phenylmethylsulfonyl fluoride (a serine protease inhibitor); (3) n-ethylmaleimide and various nonselective peptidase inhibitors (e.g., EDTA, o-phenanthroline, bacitracin); (4) benzamidine ($2x\ 10^2$ mol/L); (5) a mixture of peptidase inhibitors including amastatin, captopril, phosphoramidon); (6) a mixture of peptidase inhibitors such as actinonin (6 uM), arphamenine B (6 uM), bestatin (10 uM), captopril (10 uM) and thirophan (0.3 uM); and (7) one or more of the protease inhibitors that are useful for treating HIV infection (e.g., ritonavir, saquinavir, indinovir).

The antigen compositions of the invention can be preserved at reduced temperatures (e.g., frozen to prevent bacterial growth) or alternatively, can be lyophilized for prolonged storage. Preferably, the antigen compositions are formulated to contain an amount of disease-associated antigens for mixing with a single dose of dendritic cells. In general, each dendritic cell contains approximately 100,000 MHC sites for binding to antigenic peptides. Accordingly, a single dose of dendritic cells is introduced into an excess of disease-associated antigens to drive the reaction to completion, i.e., to ensure that as many disease-associated antigens as possible are

loaded onto the MHC sites of the dendritic cells. Thus, a sufficient number of disease-associated antigens are allowed to react with each dendritic cell to fill between 300 and 300,000 Class I sites and thereby elicit a specific immune system response to the presented antigen. It is well known that as few as three hundred MHC sites occupied by a particular antigenic peptide are sufficient to elicit an immune response. In general, this is accomplished by incubating the eluate from ten-fold to one hundred-fold disease effector cells with one-fold number of dendritic antigen presenting cells.

According to yet another aspect of the invention, an alternative process for producing an antigen product for use in enhancing an immune response is provided. The process involves two steps: (a) acidifying a preparation containing a plurality of disease effector cells for a period of time sufficient for the disease effector cells to release disease-associated antigens without immediately lysing the cells; and (b) neutralizing the acidified preparation to form the product. The disease effector cells are obtained from peripheral blood and include, for example, malignant T cells, malignant B cells, T cells or B cells which mediate an autoimmune response, T cells or B cells which mediate transplanted tissue rejection, and virally, bacterially or protozoally infected disease effector cells which express on their surface viral, bacterial or protozoan proteins and/or peptides. Preferably, the disease effector cells express on their surface an antigen that is associated with (bound to) an MHC Class I protein, an MHC Class II protein or a heat shock protein that is capable of transporting peptide to or from an MHC site. More preferably, the disease effector cells are isolated from peripheral blood prior to acidification. In the preferred embodiments, the disease effector cells are T cells or B cells. More preferably, the disease effector cells are T cells, preferably within a single family. In the most preferred embodiments, the T cells are of a single clone.

The acidification step of the foregoing process is based upon published procedures. See, e.g., Storkus, W., *et al.*, *J Immunotherapy* 14:94-103 (1993) and L. Zitvogel, *et al.*, *J Exp Med* 183:87-97 (1996). An exemplary protocol for acid eluting antigens from the MHC Class I sites of antigen presenting cells is provided in the Examples. In the preferred embodiments, the acid elution procedure is performed

at room temperature. In general, the acidification step involves subjecting the preparation of disease effector cells to a pH of between about pH 2 and pH 6 (preferably between pH2 and pH 4) for between about 0.5 to about 20 minutes. In the preferred embodiments, acidification involves subjecting the preparation to a pH of about 3.3 for about one minute. Thereafter, the cell preparation is neutralized in accordance with standard practice (e.g., by washing pelleted or flask-adherent cells with buffered tissue culture medium), and the eluted peptides preferably are further concentrated (e.g., by chromatography and/or lyophilization). Performing the acidification step under these conditions, results in release by the disease effector cells of their disease-associated antigens without immediately lysing the disease effector cells. Optionally, the acid-eluted antigen preparation is divided into aliquots, each aliquot containing an amount of antigen sufficient for mixing with a single dose of dendritic cells to enhance the immune system. Preferably, the aliquots are lyophilized to facilitate storage and shipping. Although not intending to be bound by a particular theory, it is believed that incubation of cells a pH 3.3 in citrate-phosphate buffer denatures Class I complexes, resulting in the release of beta2 microglobulin and previously Class I-bound peptides into the extracellular media (Storkus, W., *et al.* (*J Immunotherapy* 14:94-103 (1993)). Because the mild pH treatment does not immediately lyse the disease effector cells, the cells regenerate their Class I peptide complexes in culture, thereby providing a mechanism whereby multiple batches of disease-associated antigens can be harvested from the disease effector cells in culture.

In a particularly preferred embodiment, dendritic cells or other antigen presenting cells, are subjected to the above-described acid elution/neutralization protocol prior to contacting the cells with the disease-associated antigens. In this manner, the MHC molecules of the dendritic cells are emptied of their endogenous peptides prior to exposure to the disease-associated antigens, thereby increasing the number of empty MHC molecules available for association with the disease-associated antigens and rendering the acid-eluted dendritic or other antigen presenting cells more efficient antigen presenting cells, presumably, by providing an increased number of empty MHC sites into which the disease-associated antigens can be loaded. It is

believed that the above-described acid elution/neutralization protocol also results in release of B2-microglobulin from the MHC molecules. Accordingly, it is preferred that the acid-eluted, neutralized antigen presenting cells be incubated with B2-microglobulin prior to, or concurrent with, contacting the cells with disease-associated antigens to increase the efficiency of antigen presentation by the acid-eluted, neutralized antigen presenting cells. Further, in the preferred embodiments, the acid-eluted, neutralized dendritic cells are contacted with the disease-associated antigens at a temperature that is less than physiological temperature to further stabilize the empty MHC sites of these antigen presenting cells.

In a related aspect of the invention, a product for enhancing an immune response is disclosed. The product is produced by the process of: (a) acidifying a preparation containing a plurality of disease effector cells for a period of time sufficient for the disease effector cells to release disease associated antigens without immediately lysing the cells; and (b) neutralizing the acidified preparation to form the product. Preferably, the disease effector cells are obtained from peripheral blood. As disclosed above in reference to the process, the product can be an aliquoted product in which each aliquot contains an amount of neutralized product sufficient to mix with a single dose of dendritic cells for administration to a subject to enhance the subject's specific immune system response to the presented antigen.

The specific examples presented below are illustrative only and are not intended to limit the scope of the invention.

Example 1

Preparation of Dendritic Cells

Various methods have been reported for the isolation of dendritic cells from, e.g., human peripheral blood, bone marrow and spleen cells. For example, PCT Application No. PCT/US93/06653 having publication Number WO 94/02156, entitled "Methods for Using Dendritic Cells to Activate T Cells" (Engleman *et al.*, hereinafter "WO 94/02156") describes methods for isolating dendritic cells from human blood and for using the isolated dendritic cells to present antigens for the induction of an antigen-

specific T-cell-mediated immune response. More recently, methods have been reported for the isolation of precursor dendritic cells and their expansion *in vitro*. For example, PCT Application No. PCT/US93/03141 having publication Number WO 93/20185, entitled "Method for in vitro Proliferation of Dendritic cell Precursors and their use to produce Immunogens" (Steinman *et al.*, hereinafter WO 93/20185) describes methods for isolating dendritic cell precursors from human blood, expanding the isolated cell precursors *in vitro* in the presence of GM-CSF, and pulsing the expanded cell precursors with peptide antigen *in vitro* to obtain peptide-loaded dendritic cells that are suitable for inducting an immune system response. The following procedures for isolating and culturing dendritic cells/dendritic cell precursors from human peripheral blood are based upon the protocols for culturing such cells that are described in WO 94/02156 and WO 93/20185.

(A) Isolation and Culture of Dendritic Cell Precursors obtained from Human Blood

The procedure described herein is adapted from the isolation and culturing protocols provided in WO 93/20185 (Steinman *et al).*. Briefly, blood mononuclear cells are isolated by sedimentation in standard dense medium, such as Lymphoprep (Nycomed, Oslo). The isolated mononuclear cells are depleted of cells that are not dendritic cell progenitors. For example, these contaminants are coated with monoclonal antibodies to CD3 and HLA-DR antigens and depleted on petri dishes coated with affinity-purified, goat anti-mouse IgG ("panning"). Approximately 10^6 cells in one ml of culture medium are plated in 16 mm diameter plastic culture wells (Co-star, New York). The medium (e.g., RPMI-1640) is supplemented with typical growth nutrients (e.g., 50 uM 2-mercaptoethanol, 10 mM glutamine, 50 μg/ml gentamicin, 5% serum from cord blood without heat inactivation or 5% fetal calf serum (with inactivation)) and human recombinant GM-CSF (preferably 400 U/ml). Optionally, serum-free medium that is appropriate for mammalian cell culture can be used. Every second day thereafter and for a total of 16 days, the cultures are fed by removing 0.3 ml of the medium and replacing this with 0.5 ml of fresh medium supplemented with the cytokines. Preferably, the cells are cultured n the presence of

additional cytokines, such as IL-4, IL-12, IL-1 alpha, TNF alpha, Il-3, FGF and/or LAF. Typically, these additional cytokines are added during the last 24 hours of dendritic cell culturing. These same cytokines optionally are added during administration of the antigen-loaded dendritic cells to the subject.

Characteristic proliferating dendritic cell aggregates (termed "balls" by Steinman *et al.* in WO 93/20185) appear by the fifth day, as evident by examination with an inverted phase contrast microscope. The balls expand in size over the course of a week. Some balls appear in the original wells, but typically these do not enlarge to the same extent as the non-adherent wells. The wells are subcultured, e.g., one well is split into two or three wells, as cell density increases.

Two alternative approaches can be used to isolate mature dendritic cells from the growing cultures. The first method involves removing cells that are non-adherent and separating the balls from non-balls by 1 g sedimentation. Dendritic cells then are released in large numbers from the balls over an additional one or two days of culture and the mature dendritic cells are isolated from the non-balls by flotation on dense metrizamide as previously described (Freudenthal and Steinman, *Proc Natl Acad Sci USA* 87:7698-7702 (1990)). Alternatively, mature dendritic cells are isolated by harvesting the non-adherent cells when the balls are very large. The cells are then left on ice for 20 minutes, resuspended vigorously with a pipette to disintegrate the balls, and the mature dendritic cells are floated on metrizamide columns.

GM-CSF reportedly is an essential cytokine for the development of dendritic cell balls. IL-4 and to a lesser extent, IL-12, also facilitate dendritic cell culture. The addition of TNF alpha at 10-50 U/ml increases dendritic cell yields approximately two-fold. Starting with 60 ml of blood culturing in the presence of GM-CSF alone, the yield of mature dendritic cells is between 6 to $12x10^6$ cells, representing 40-80 percent of the cells. Alternatively, a sufficient number of dendritic cells for achieving the purposes of the invention can be isolated from blood without prior culture (see below, "Isolation of Dendritic Cells from Human Blood").

Other sources of dendritic cell progenitors, e.g., bone marrow, spleen cells, and fetal or umbilical cord blood, also can be used. For example, PCT application number

PCT/US91/01683, publication number WO 91/13632, entitled "Idiotypic Vaccination Against B Cell Lymphoma" ("Hohlen *et al.*") describes a protocol for isolating dendritic cells from spleen. The Hohlen *et al.* protocol is based upon the method previously reported by Steinman and Cohen, *J Exp Med* 139:380-397 (1974).

(B) Isolation of Dendritic Cells from Human Blood

The procedure described herein is adapted from the isolation and culturing protocols provided in WO 94/02156 (Engleman *et al.)*. Although dendritic cells are found in both lymphoid and nonlymphoid tissues, the most readily accessible source of dendritic cells in man is peripheral blood, which contains less than about 1 dendritic cell per 100 disease effector cells. To obtain a sufficient number of dendritic cells directly from blood without necessitating dendritic cell culture, a disease effector cell concentrate is prepared in accordance standard leukapheresis practice. In general, approximately two billion disease effector cells are collected during leukapheresis. Thus, assuming that the dendritic cells represent one percent of the total disease effector cell population collected by leukapheresis, approximately 20 million dendritic cells are present in the leukapheresis disease effector cell concentrate. As discussed below, this number of cells is sufficient to perform multiple treatments in accordance with the methods disclosed herein. In addition, further culture of these dendritic cells can be performed to increase further the total number of dendritic cells for therapy. For the *in vivo* priming of an immune system response, a highly purified dendritic cell population (of at least about 80%, preferably of at least about 90%) is recommended.

The number of dendritic cells present in blood and, hence, in a leukapheresis disease effector cell concentrate, can be increased by administering one or more agents which stimulate hematopoiesis prior to photopheresis or leukapheresis. Such agents include G-CSF, GM-CSF and may include other factors which promote hematopoiesis. The amount of hematopoietic agent to be administered is determined by monitoring the cell differential of subjects to whom the factor(s) are administered. Typically, dosages of cytokine agents, such as G-CSF and GM-CSF, are similar to the dosages of these agents that are administered to treat subjects recovering from treatment with cytotoxic agents. Preferably, GM-CSF or G-CSF is administered for 4 to 7 days at standard

doses prior to removal of the source tissue (e.g., blood, bone marrow) to increase the proportion of dendritic cells. (Editorial, Lancet 339:648-649 (March 14, 1992)). Exemplary dosages are provided in Steinman *et al.* (WO 93/20185). For example, dosages of G-CSF of 300 μg daily for 5 to 13 days and dosages of GM-CSF of 400 μg daily for 4 to 19 days reportedly result in a significant increase in dendritic cell precursors *in vivo*. It is believed that GM-CSF activates the dendritic cells *in vivo*, thereby causing the dendritic cells to release their own cytokines which further activate $CD8^+$ cells *in vivo*. Accordingly, cytokines in addition to GM-CSF (e.g., IL-12 and IL-4) optionally are coadministered to the subject to facilitate this process.

In general, human peripheral blood mononuclear leukocytes (PBML) are isolated from blood samples, particularly buffy coat or leukocytes prepared by, for example, aphereses (optional), Ficoll Hypaque gradient centrifugation followed by Percoll density centrifugation. Twenty-five to five-hundred milliliters of blood that is processed by Ficoll Hypaque gradient centrifugation and Percoll density centrifugation will provide a sufficient number of dendritic cells for further expansion *in vitro*. The high buoyant density fraction (HD) contains the T cells, B cells and dendritic cells, whereas the monocytes are contained in the low buoyant density (LD) fraction. Centrifugation of the HD fraction in Nycodenz/Nycoprep (Nycomed Pharma, Oslo, Norway) separates the dendritic cells (present in the LD fraction) from the T and B cells (present in the HD fraction). The dendritic cells optionally are further enriched using additional protocols (described below).

Alternatively, dendritic cells are isolated using procedures which involve repetitive density gradient centrifugation, positive selection, negative selection, or a combination thereof. For example, negative selection of dendritic cells can be accomplished by panning using antibodies to remove nondendritic cells to result in a preparation containing approximately 80-90% dendritic cells. Alternatively, positive selection can be performed in which affinity chromatography is employed wherein antibodies to dendritic cell surface markers are used as the affinity chromatography ligand to remove dendritic cells from a complex mixture. Exemplary antibodies that are useful for negative and/or positive selection are described in WO 94/02156.

Briefly, human dendritic cells can obtained from buffy coats using the following procedure. Peripheral blood mononuclear leukocytes (PBML) are isolated by Ficoll-Hypaque gradient centrifugation (Bouyam, *Scand J Clin Lab Invest* 21:21-29 (1968)). Blood dendritic cells optionally are further separated by, for example, the methods described in WO 94/02156. (See, in particular, WO 94/02156, Fig. 1, for an overview of the separation process). Briefly, PBML are separated into LD and HD fractions in a four-step discontinuous Percoll gradient (Pharmacia Uppsala, Sweden) (Markowicz and Engleman, *J Clin Invest* 85:955-961 (1990)). The HD fraction containing the dendritic cells is collected and cultured in culture media in Teflon vessels for 16-28 hours at 37°C. Thereafter, the cells are centrifuged over a Nycodenz/Nycopret discontinuous gradient (Nycomed Pharma, Oslo, Norway). The dendritic cells are contained entirely in the LD fraction and occupy approximately 30-40% of the total cell population. This partially purified dendritic cell population can be used for T cell priming and activation experiments *in vivo* or *in vitro*.

In the preferred embodiments, the dendritic cells are further purified for *in vivo* applications. Further purification of the dendritic cell population is achieved by performing a second round of Nycodenz/Nycoprep centrifugation and collecting the LD fraction obtained therefrom. The LD fraction contains approximately 80-90% dendritic cells. Alternatively, the LD fraction following the first Nycodenz/Nycoprep step is incubated with antibody-coated petri dishes to remove $CD3^+$, $CD14^+$, $CD16^+$, and $CD20^+$ cells to obtain a nonadherent cell population containing between approximately 80-90% dendritic cells. In general, these procedures produce a yield of $1\text{-}2.5\text{x}10^6$ cells from about 400-500 ml of whole blood.

Assessment of dendritic cell purity following enrichment is determined by staining with an anti-HLA-DR antibody (e.g., an anti-MHC Class II antibody such as CA141) which is conjugated to a detectable reagent (e.g., fluorescein), and an antimonocyte antibody such as phycoerythrin-conjugated anti-CD14. Cytofluorographic analysis of the cell population is assessed by fluorescence-activated cell sorters. $HLA\text{-}DR^+$ $CD14^-$ cells represent the dendritic cell population. In general, dendritic cells are readily distinguished from other PBML on the basis of their high

levels of expression of MHC-Class II determinants and their lack of CD14 expression. Further definitive analysis of the cell population is accomplished by determining whether the putative dendritic cells are also negative for a variety of known T cell and B cell markers and are positive for a variety of known dendritic cell markers (discussed above).

Example 2

Preparation of Disease-Associated Antigens for *In Vitro* Loading

Disease-associated antigens for *in vitro* loading into dendritic or other antigen presenting cells of the invention are prepared in accordance with procedures known to those of ordinary skill in the art. The procedure described herein is based upon the acid elution protocol described in Zitvogel, L. *et al. J Exp Med* 183:87-97 (1996). (See also, e.g., Storkus, W. *et al. J Immunotherapy* 14:94-103 (1993) for buffer and reagent preparation for the acid elution procedure).

Disease effector cells (approximately 1-5x10^9 cells) obtained from the extracorporeal blood stream are washed three times in HBSS (GIBCO-BRL), and the cell pellet is treated with mild acid buffer. Briefly, 10 ml of citrate-phosphate buffer, pH=3.3, is added at room temperature, and the cell pellets are immediately resuspended by, for example, pipetting, and centrifuged for 5 min. at 1,000 g. The cell-free supernatant is harvested, and peptides in the acid-extracted supernatants are concentrated, e.g., on activated SepPak C18 cartridges (Millipore Corp., Bedford, MA). The bound material is eluted with 2-3 ml of 60% acetonitrile in water and lyophilized to near complete dryness (e.g., 20-50 µl). The peptides are then reconstituted in 1 ml HBSS (GIBCO-BRL) and stored frozen (e.g., at -70°C) until loading onto dendritic or other antigen presenting cells of the invention. In general, one million dendritic cells are allowed to react with peptides derived from 10^8 to 10^9 effector cell equivalents in a total volume of 1-2 ml of dendritic cell culture medium (e.g., overnight incubation at 37°C, 5% carbon dioxide). In the preferred embodiments, the loading reaction is performed at a temperature less than physiological temperature (e.g., between about 22°C and 27°C).

Example 3

Loading Disease-Associated Antigens onto Dendritic Cells

In the preferred embodiments, dendritic cells are introduced into the extracorporeal blood stream at any stage during photopheresis. More preferably, the dendritic cells are acid-eluted or agent treated as discussed above prior to introduction to the extracorporeal blood stream. Acid elution or agent treatment (such as using 8-MOP/UVA) of the dendritic cells induces release by the cells of peptides that may have become associated with their MHC Class I molecules during cell isolation and/or culture. Accordingly, in the most preferred embodiments, the acid-eluted dendritic cells are introduced to the extracorporeal blood stream at a temperature less than physiological temperature (between about 22°C and 27°C) to enhance dendritic cell empty MHC stability and minimize enzymatic antigenic peptide degradation.

Introduction of the dendritic cells into the extracorporeal blood stream is accomplished using standard injection ports (i.e., ports on the intravenous tubing sets) known to those of ordinary skill in the art. Preferably, between $1x10^6$ and $10x10^6$ dendritic cells constitute a single dose for injection into the subject. However, as discussed above, a number of dendritic cells to support multiple doses (e.g., 100-fold the number of cells for a single dose) can be introduced into the extracorporeal blood stream to prepare a stock preparation of antigen-loaded dendritic cells for storage and subsequent booster immunizations. In the preferred embodiments, this preparation of antigen-loaded dendritic cells is stored in aliquots containing a single dose for re-injection into the subject. In general, in a single dose of antigen-loaded dendritic cells, 300 to 300,000 MHC sites per cell are occupied by disease-associated peptides. More preferably, between about 1000 and 200,000 MHC site per cell are occupied by disease-associated peptides.

Following injection of the dendritic cells, the disease-associated antigens which are present in the extracorporeal blood stream following their release from the disease effector cells are loaded onto the MHC sites of the exogenously added dendritic cells. The photopheresis process steps (e.g., centrifugation) provide sufficient agitation and mixing of the dendritic cells and the disease effector cells from which the antigens are

derived to facilitate rapid loading of the release antigens onto the dendritic cell MHC sites, thereby further reducing the likelihood of enzymatic degradation of the release antigens.

Alternatively, the treated blood containing the disease-associated antigen is loaded into the MHC sites of dendritic cells *in vitro*, as opposed to introducing the cells to the extracorporeal blood stream. Preparation of the disease-associated antigens by acid elution of disease effector cells is described in Example 2, above. The antigen compositions of the invention which are useful for this purpose are disease-associated antigens which have been released from disease effector cells that are contained in blood. The compositions contain a detectable amount of a photoactivatable agent (e.g., a psoralen), such as the amount that would be present in the blood of a subject who has been subjected to photopheresis therapy. The composition is prepared by aliquoting the photopheresed blood into portions which contain an amount of disease-associated antigens suitable for mixing with one or more doses of dendritic cells. Preferably, the antigen composition for enhancing an immune response is prepared by acidifying a preparation containing a plurality of disease effector cells (e.g., disease effector cells contained in the extracorporeal blood stream) for a period of time sufficient for the disease effector cells to release the disease-associated antigens without lysing the disease effector cells. The preparation is neutralized prior to loading the released antigens onto the MHC sites of the dendritic cells.

In general, the disease-associated antigens which are prepared in accordance with the methods of the invention (by photopheresis alone or together with acid elution of antigens) are loaded onto dendritic cells in accordance with the procedures described by Steinman *et al.* (WO 93/20185). Briefly, dendritic cells prepared as described above are plated at a concentration of approximately $1x10^5$ cells per well of a 24-well plastic culture plate. For antigen preparations in which the peptides are intended to be directly loaded into the MHC sites (without phagocytosis), mature dendritic cells are preferred. The cells are incubated and culture medium (e.g., RPMI 1640) preferably containing additional nutrients (e.g., 5% fetal calf serum), and

GM-CSF (preferably at 30 U/ml). Preferably, the dendritic cells are subjected to acid elution (as described in Example 2, above), washed and placed in an appropriate loading medium at less than physiological temperature prior to contacting the dendritic cells with the disease-associated antigens. Loading the dendritic cells at a temperature less than physiological temperature enhances empty MHC stability and thereby maximizes the number of MHC sites available for association with the disease-associated antigens. Reduced temperature loading also minimizes the likelihood of enzymatic digestion of the released peptide antigens.

The disease-associated antigen preparation of the invention is added to the dendritic cell cultures and the cultures are incubated with the antigen for several hours or for sufficient time to allow the dendritic cells to present the antigen in a form which is recognized by T cells. Preferably, the cultures are incubated at a temperature less than physiological temperature to maximize the number of empty MHC sites available for antigen loading. Following loading of the disease-associated antigens into the dendritic cell MHC sites, the cells are collected from the culture, washed extensively and are used to immunize the subject. Of course, a known control antigen can be included in the preparation as a control and a portion of the collected cells can be devoted to a quality control assay to determine (1) the viability of the dendritic cells and/or (2) the functional activity of the antigen-loaded dendritic cells with respect to their ability to induce an antigen-specific immune response *in vitro* (e.g., a cytotoxic T cell assay) or *in vivo*. For example, the peptide-loaded dendritic cells can be injected subcutaneously into a mouse in an amount sufficient to induce an immune response to the known control antigen to estimate the efficiency of antigen loading for the tested cells. In the preferred embodiments, the antigen-loaded dendritic cells are irradiated (3000 rads gamma irradiation) before injection (preferably, i.v., or i.d. injection). More preferably, the antigen-loaded dendritic cells are coadministered with one or more cytokines (e.g., GM-CSF, IL-12, IL-4) to further enhance a specific immune response to the disease-associated antigen. (See, e.g., Zitvogel *et al.*, *J Exp Med* 184:87-97 (1996) which reports that co-administration of peptide-pulsed dendritic cells with low doses of IL-12 may favor the priming of tumor-specific T cells).

Optional booster immunizations are conducted in accordance with standard practice using the above-described disease-associated antigen-loaded dendritic cell preparation. In general, the cells are tested for activity, viability, toxicity and sterility prior to administration to the subject. The cytotoxic activity of the peptide-loaded dendritic cells can be determined in a cytotoxic T cell assay, e.g., a chromium release assay, using target cells that express the appropriate MHC molecule, in the presence and absence of the disease-associated peptide or other known peptide control that is known to be capable of loading onto dendritic cells and invoking a cytotoxic T cell response *in vitro*. (See, e.g., WO 94-20127 and Zitvogel, L., *et al.*, *J Exp Med* 183:87-97 (1996)). Zitvogel, *et al.* also describe an animal model for testing the ability of antigen-loaded dendritic cells (pulsed with acid-eluted peptides derived from autologous solid tumors) to inhibit tumor progression *in vivo*. The Zitvogel animal model is adapted for use in the current invention to further optimize the procedures for obtaining the disease-associated antigens of the invention, loading these antigens into the MHC sites of the dendritic or other antigen presenting cells of the invention and selecting the optimum dose and booster frequency for the subject. However, in contrast to the literature reports, increased serum levels of $CD4^+$ and/or $CD8^+$ T cells (rather than solid tumor size) are used as indicator(s) of disease inhibition.

Cell viability is determined by the exclusion of trypan blue dye by live cells. Cells are tested for the presence of endotoxin and for bacterial or fungal contamination by conventional methods known to those of ordinary skill in the art. Cells which have passed these safety and activity criteria are washed and placed in the appropriate solution (e.g., an infusion solution such as Ringer/glucose lactate for i.v. infusion) and administered to the subject.

Additional methods for pulsing dendritic cells with antigen and/or procedures for using antigen-loaded dendritic cells to induce a cytotoxic T cell response *in vitro* or *in vivo* are described in WO 93/20185, WO 94/21287, WO 94/02156; Mayordomo, J. *et al.*, *Nature Medicine* 1(12):127-1302 (1995); and Hsu, F. *et al.*, *Nature Medicine* 2(1):52-58 (1996). See, also, WO 94-21287, WO 94-20127 and Hsu, F. *et al.*, *Nature Medicine* 2(1):52-58 (1996) for exemplary protocols for irradiating antigen-loaded

dendritic cells to prevent cell proliferation when the cells are reintroduced into the subject.

Example 4

Demonstrated Efficacy of Photopheresis/Dendritic Cell Treatment

To demostrate the effectiveness of combining extracorporeal treatment of disease effector cells and the addition of dendritic cells, murine 2B4.11 tumorigenic T cells were treated as described above using 8-MOP and UVA. The 2B4.11 tumor cells were derived by hybridizing or combining two original cell types: normal AKR mouse T cells with a BW5147 mouse malignant T cells. The AKR parental cell provides specific antigens, including a T cell receptor, which can apparently serve as a tumor specific antigen, to be targeted by an induced anti-tumor immunologic reaction. The BW5147 parental cell contribution permits the 2B4.11 cells to act like a cancer cell, dividing without check until they kill the animal.

Following 8-MOP/UVA treatment, dendritic cells were added to the treated cell mixture.

Two groups of 5 test mice were vaccinated with 5 million 2B4.11 cells that had been inactivated with 8-MOP/UVA treatment and mixed with dendritic cells (DPAC). The 8-MOP/UVA irradiated tumorigenic cells were shaken overnight with 200,000 dendritic cells (a 25 to 1 ratio), to maximize cell-to-cell contact between the DAPCs and the 2B4.11 cells. One group of cells (irradiated 2B4.11 plus DAPCs) was incubated at 23° C, to maximize stability of empty class I MHC molecules on the DAPCs. The other group of cells was incubated at 37° C, to maximize normal cellular metabolism. The combined irradiated 2B4.11/DAPC cell mix was injected into the test mice one week prior to challenging the animals with viable, tumorigenic 2B4.11 cells.

In Figure 1, reading from left to right, tumor growth is first depicted in five control mice, which received only skin injections of tumor cells on day 0 and no anti-tumor vaccination. All of these mice had visible tumors by day 8, which progressively grew until day 21, the last day of observation in these experiments.

Of the vaccinated mice, all ten (both groups that received DAPCs) developed tumors that grew more slowly than those in the control mice, or did not grow at all. Three of the mice receiving the 23°C cell mix and two of the mice receiving the 37°C cell mix did not grow tumors at all. The other two mice in the 23°C group had small tumors which stopped enlarging after day 11. One of the other mice in the 37°C group also had a very small tumor which stopped growing by day 11. Of great interest, two of the other mice in the 37°C group developed small, slowly growing tumors, which then completely resolved.

This data demonstrates that direct contact between 8-MOP/UVA irradiated tumorigenic cells and DAPCs leads to formation of an effective cellular vaccine, which not only prevents or slows tumor growth, but reverses growth of certain tumors. The Figure 1 demonstrates that the combination of genetically identical dendritic antigen presenting cells (DAPCs) plus 8-MOP/UVA pretreated murine 2B4.11 tumorigenic T cells constitutes an effective vaccine against the specific tumor. (It has previously been shown that inoculation of mice with the tumorigenic 2B4.11 cells kills the mice within 40 days, unless the animals have been successfully vaccinated against the tumor). Alternatively, the antigen loaded dendritic cells can be isolated following mixing. Further, a membrane partition, for example a .45 micron filter, can be place between the dendritic cells and the disease effector cells to allow passage of the antigens to the dendritic cells but keeps the two cell population separated.

Example 5

Demonstrated Efficacy of Dendritic Cell Therapy

Figure 2 shows results that demonstrate the impact of exogenously supplied dendritic antigen presenting cells, that are supplied without prior contact with treated disease effector cells, on tumor growth. Large tumors grew in control animals, with initial appearance of tumor on day 7 and continuous enlargement through the observation period to day 28. Vaccination with DAPCs cultured overnight at either 23°C or 37°C prevented tumor growth, respectively, in 4 and 3 mice out of both groups of 5, and reversed initial limited growth in the remaining tumors. This effect is

substantial, but does not lead to the specific immunologic memory which follows vaccination with the 2B4.11/DAPC mix shown in the Figure 1. For example, although not shown in these graphs, if a second 2B4.11 tumor is administered after the resolution of the tumors, immunoprotection is only seen in the group getting the combination vaccination of 2B4.11 mixed with DAPCs.

Example 6

Demonstrated Increase in MHC Expression Following Photopheresis

Figure 3 shows the impact of 8-MOP/UVA on Class I expression. Mean fluorescence channel quantifies the amount of Class I protein on the cell surface, as identified with a fluorescent antibody against the Class I protein. Normally, at least 200,000 Class I molecules are displayed, as shown by a value of 2.4 in the control cell population. After exposure to 8-MOP/UVA (1 joule per cm2 UVA and 200 ng/ml of 8-MOP) and overnight incubation, Class I expression doubles. Since this massive increase in Class I expression is prevented by emetine, it results from new protein synthesis.

It should be understood that the preceding is merely a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All references, patents and patent publications that are identified in this application are incorporated in their entirety herein by reference.

Claims

1. An improved method of extracorporeally treating the blood, cells or tissues of a diseased subject to enhance an immune system response to one or more disease-associated antigens, wherein the blood, cells or tissues contains disease effector cells that have been naturally stimulated as a consequence of the disease state to express one or more disease-associated antigens and wherein the extracorporeal treatment induces release by the disease effector cells of the disease-associated antigens, the improvement comprising:

(a) introducing antigen presenting cells into the treated blood, cells or tissue to form a therapeutic mixture, wherein the antigen presenting cells are introduced under conditions to enhance contact between the antigen presenting cells and the antigens released from the disease effector cells; and

(b) reinfusing the therapeutic mixture into the diseased subject, wherein the antigen presenting cells are present in an amount sufficient to enhance the immune system response of the diseased subject to the one or more disease-associated antigens relative to an immune system response that would have been induced had the extracorporeal treatment been performed in the absence of the antigen presenting cells.

2. The method of claim 1, wherein the antigen presenting cells are dendritic cells.

3. The method of claim 1, wherein the extracorporeal treatment comprises treating the blood, cells or tissues of the diseased subject with an agent that increase MHC expression on the treated cells.

4. The method of claim 3, wherein the extracorporeal treatment comprises treating the blood, cells or tissues of the diseased subject with a photoactivatable agent to form an agent-treated blood, cell or tissue sample and photoactivating the drug-treated sample.

5. The method of claim 4, wherein the photoactivatable agent is a psoralen.

6. The method of claim 1, wherein the diseased subject has a disease that is mediated by a disease effector cell selected from the group consisting of a T cell, a B cell, a virally infected disease effector cell, a bacterially infected disease effector cell a protozoally infected disease effector cell, and a solid tumor cell.

7. The method of claim 1, wherein the disease is selected from the group consisting of a leukemia, a lymphoma, solid tumors, an autoimmune disease, graft v. host disease, transplanted tissue rejection, a viral infection that is mediated by virally infected disease effector cells, a bacterial infection that is mediated by bacterially infected disease effector cells and a protozoan infection that is mediated by protozoally infected disease effector cells.

8. The method of claim 1, wherein the disease-associated antigen is a peptide that is bound to a protein selected from the group consisting of an MHC Class I protein, an MHC Class II protein and a heat shock protein that is capable of transporting peptide to or from an MHC site.

9. The method of claim 1, wherein the dendritic cells are introduced at a stage of extracorporeal treatment selected from the group consisting of stages consisting of (1) blood collection; (2) disease effector cell isolation; (3) agent treatment; and (4) disease effector cell pooling.

10. The method of claim 1, wherein said dendritic cells and said disease effector cells are separated from each other during the contact step by a membrane that allows antigens and cytokines to pass through but does not allow the cells to pass through.

11. The method of claim 1, further comprising the step of reinfusing the therapeutic mixture in the presence of a cytokine.

12. The method of claim 11, wherein the cytokine is selected from the group consisting of GM-CSF, IL-4, TNF-ALPHA, FGF and IL-12.

13. The method of claim 2, wherein the dendritic cells have the same presentation characteristics as naturally-occurring dendritic cells.

14. The method of claim 2, wherein introducing the dendritic cells comprises introducing from about 1000 to about 100 million dendritic cells to the treated blood.

15. A composition for enhancing an immune response, comprising:

disease-associated antigens that have been released from disease effector cells contained in blood; and

a detectable amount of at least one agent selected from the group consisting of a photoactivatable agent and a protease inhibitor, wherein the composition is formulated to contain an amount of disease-associated antigens for mixing with a single dose of dendritic cells.

16. The composition of claim 15 wherein the composition is lyophilized.

17. A process for producing a product for enhancing an immune response, comprising:

(a) acidifying a preparation containing a plurality of disease effector cells for a period of time sufficient for the disease effector cells to release disease-associated antigens without lysing the cells; and

(b) neutralizing the acidified preparation to form the product, wherein the disease effector cells are obtained from peripheral blood.

18. The process of claim 17, wherein the disease effector cells are selected from the group consisting of malignant T cells, malignant B cells, T cells which mediate an autoimmune response, B cells which mediate an autoimmune response, T cells which mediate transplanted tissue rejection, B cells which mediate transplanted tissue rejection, solid tumor cells, virally infected disease effector cells which express on their surface viral proteins, bacterially infected disease effector cells which express on their surface bacterial proteins and protozoally infected disease effector cells which express on their surface protozoan proteins.

19. The process of claim 18, wherein the disease effector cells are isolated from peripheral blood prior to acidification.

20. The process of claim 19, further comprising the step of:

(c) dividing the product into aliquots, each aliquot containing an amount of product sufficient for mixing with a single dose of antigen presenting cells to enhance an immune system response.

21. A product for enhancing an immune response, the product produced by the process of claim 19.

22. In a method for photopheresis, the improvement which comprises increasing at least one of the number and activation state of dendritic cells in a subject via the steps of:

(1) administering a sufficient dosage of GM-CSF to the subject; and

(2) subjecting the subject to photopheresis,

wherein the sufficient dosage of GM-CSF is an amount and frequency of administration of GM-CSF that is sufficient to increase at least one of the number and activation state of the dendritic cells in the subject.

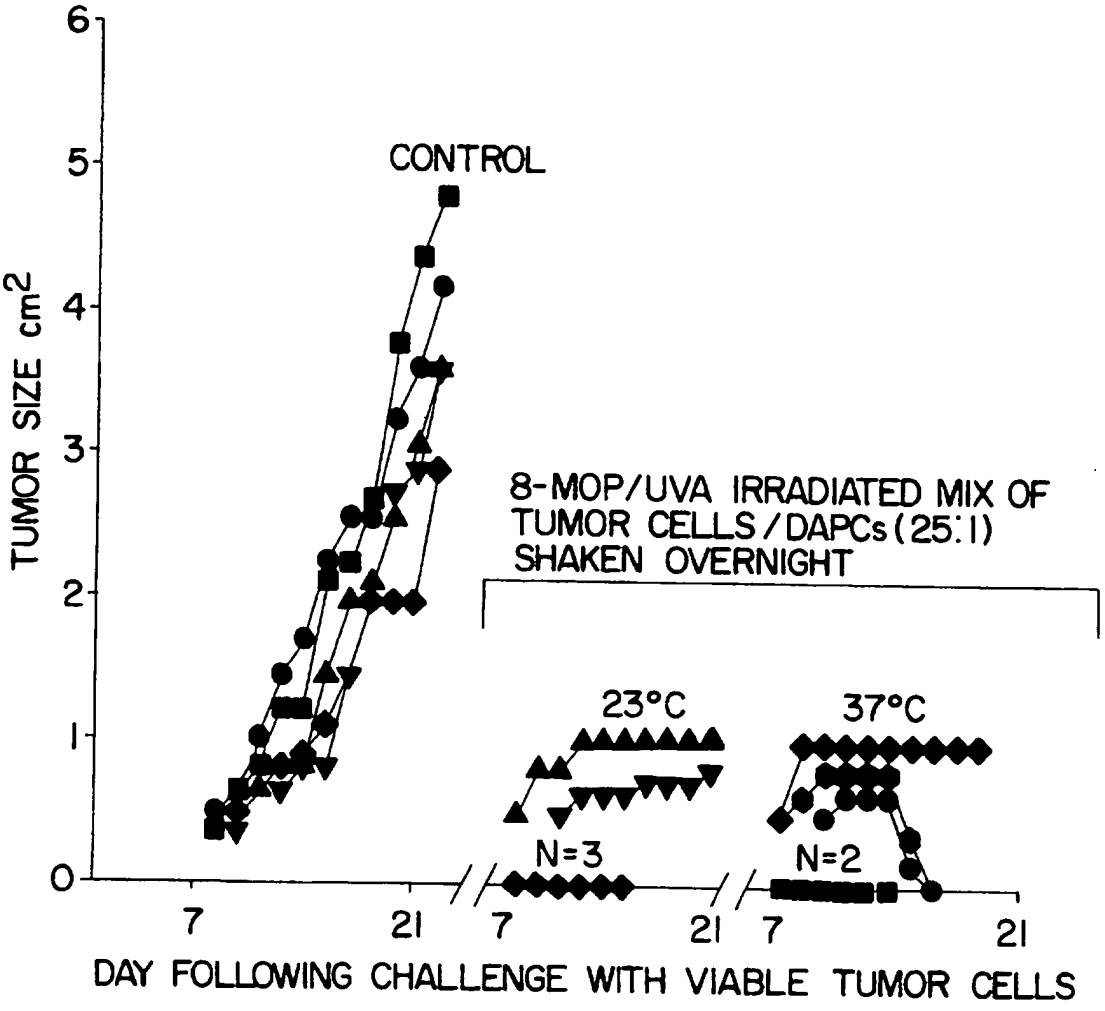
TUMOR SIZE cm2
6
5
4
3
2
1
0
CONTROL
8-MOP/UVA IRRADIATED MIX OF
TUMOR CELLS / DAPCs (25:1)
SHAKEN OVERNIGHT
23°C
37°C
N=3
N=2
7
21
7
21
7
21
DAY FOLLOWING CHALLENGE WITH VIABLE TUMOR CELLS

FIG. 1

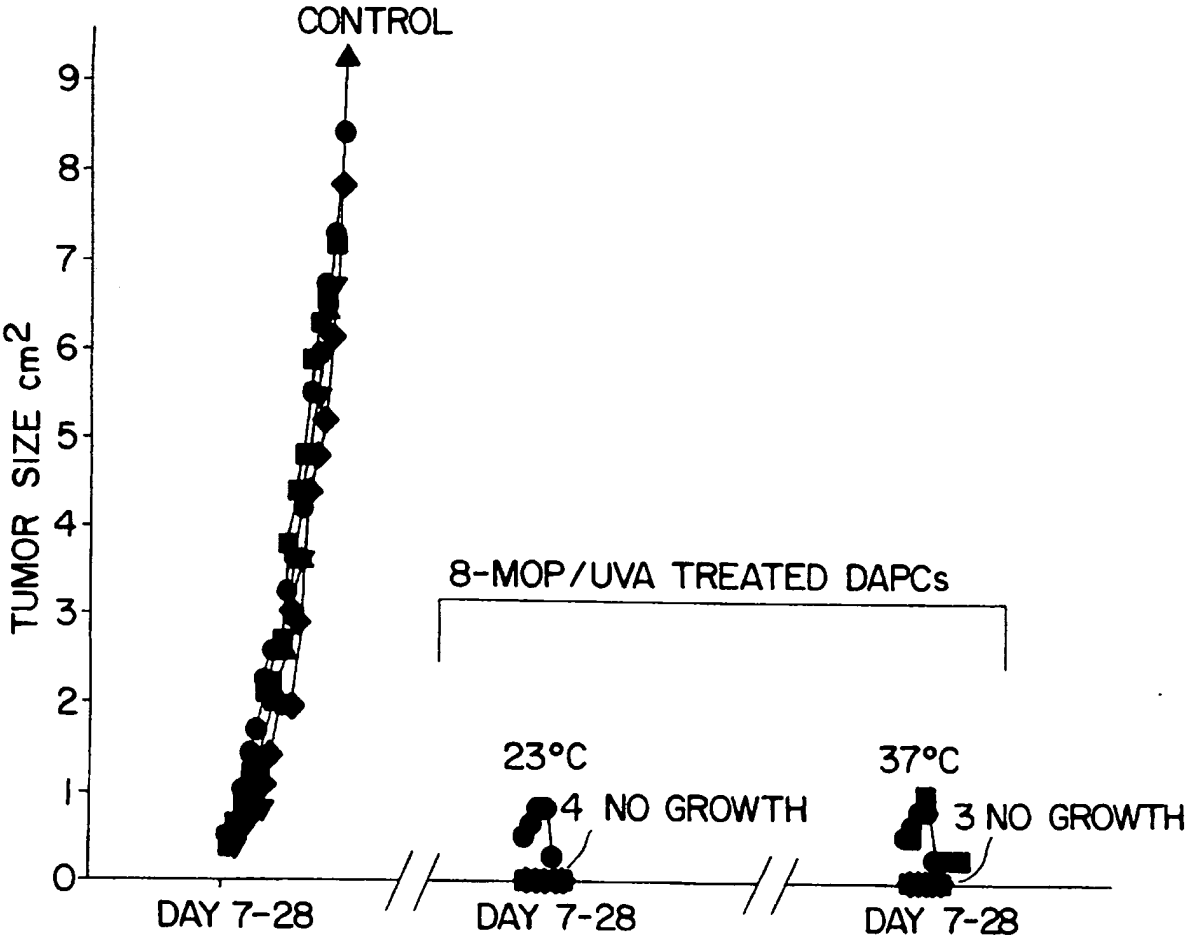
CONTROL
TUMOR SIZE cm2
0
1
2
3
4
5
6
7
8
9
8-MOP/UVA TREATED DAPCs
23°C
4 NO GROWTH
37°C
3 NO GROWTH
DAY 7-28
DAY 7-28
DAY 7-28

FIG. 2

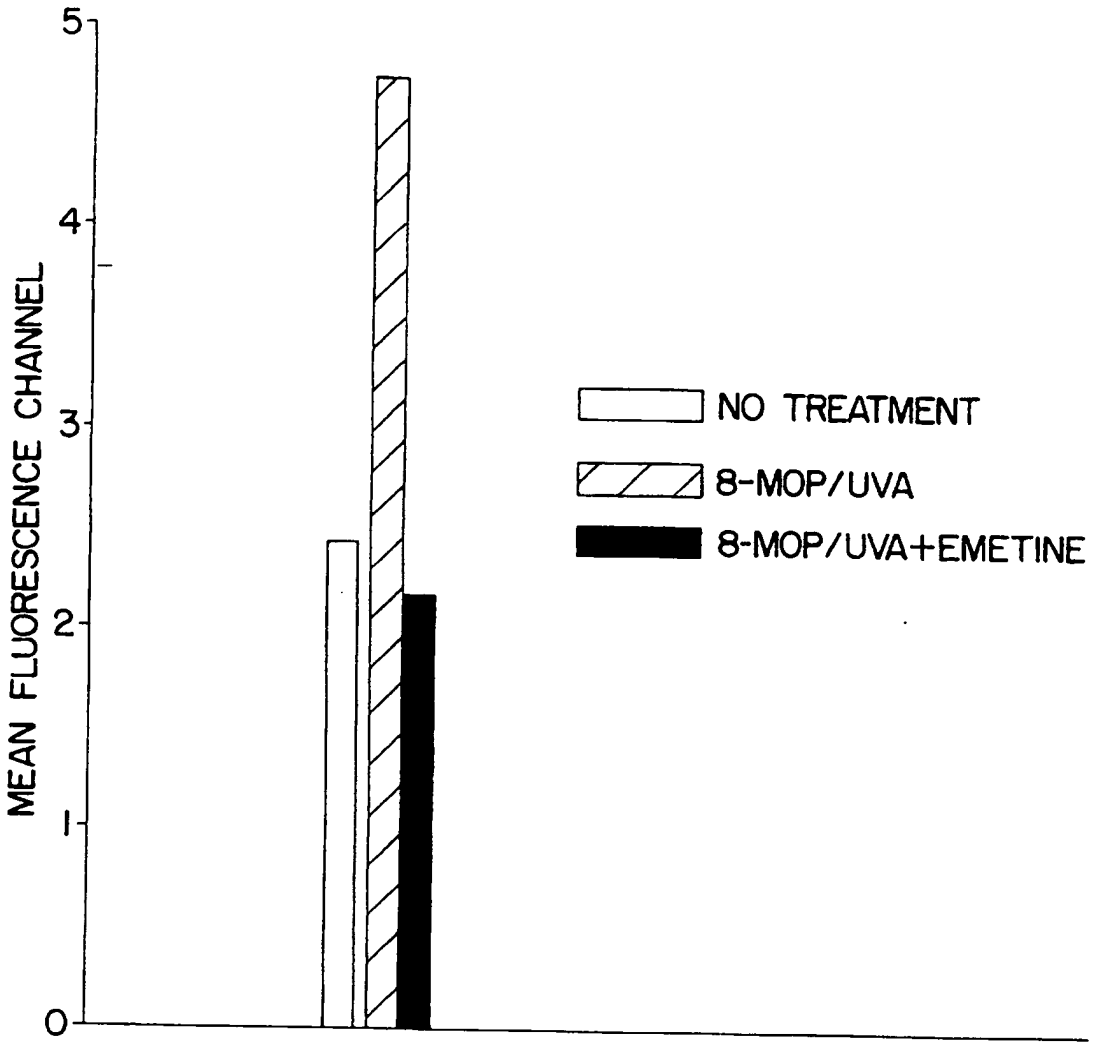
MEAN FLUORESCENCE CHANNEL
0
1
2
3
4
5
NO TREATMENT
8-MOP/UVA
8-MOP/UVA+EMETINE

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04285

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.71, 534, 577, 85.1,85.2; 435/2, 372; 514/455, 2; 530/412, 413, 418; 604/4, 6, 20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/03814 A1 (YALE UNIVERSITY) 09 February 1995, see entire document.	1-22
Y	STORKUS et al. Identification of T-cell Epitopes: Rapid Isolation of Class I-Presented Peptides from Viable Cells by Mild Acid Elution. Journal of Immunotherapy. 1993, Vol. 14, pages 94-103, see entire document.	15-21
Y	ZITVOGEL et al. Therapy of Murine Tumors with Tumor Peptide-pulsed Dendritic Cells:Dependence on T Cells, B7 Costimulation and T Helper Cell 1-associated Cytokines. Journal of Experimental Medicine. January 1996, Vol. 183, pages 87-97, see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JUNE 1997	Date of mailing of the international search report 04 AUG 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04285

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/11016 A1 (YALE UNIVERSITY) 26 May 1994, see entire document.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04285

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 1/02, 43/16, 63/00; A61K 35/14, 38/00, 38/19, 38/20, 38/21; C12N 5/08; C07K 1/14, 1/30; A61M 37/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.71, 534, 577, 85.1,85.2; 435/2, 372; 514/455, 2; 530/412, 413, 418; 604/4, 6, 20

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, EMBASE DERWENT WPI, CHEM AB, APS, search terms: author name, dendritic cell, psoralen, extracorporeal, antigen presenting, MHC expression, tumor, MHC class I, II, cytokines, acidify, Storkus, peptide, gm-csf